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A METHOD FOR THE DETERMINATION OF THE ANTICOMPLEMENTARY ACTIVITY OF HEPARIN AND RELATED COMPOUNDS

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Received for publication December 17, 1956

HEPARIN and other highly charged macromolecular compounds exhibit anticomplementary activity in a haemolytic system (Ecker and Gross, 1929; Ecker and Pillemer, 1941). A method has been devised to allow more precise quantitative comparison of this form of activity between such compounds. The present communication describes firstly, experiments designed to test the reproducibility and limits of error of the method. Secondly, its manner of application to a comparison of the activities of heparin with that of similar compounds (dextran sulphates) is also briefly outlined. The succeeding paper describes the manner in which the method has been used to define the molecular characteristics conferring varying degrees of anticomplementary activity on a series of dextran sulphates.

MATERIALS AND METHODS

Heparin and dextran sulphates.—A commercial sample of heparin, "Pularin" (Evans) batch no. F50280, was used. The anticoagulant potency was 110·9 I.U./mg. and it was found to contain 1·99 per cent nitrogen and 10·9 per cent sulphur; $[\alpha]_D^{20} = +41°$. The dextran sulphates were prepared from the same dextran fraction (a polymer estimated to consist of about 20 glucose units); one (GD) contained 12·55 per cent, and the other (I/7) 20·6 per cent sulphur.

Haemolytic system.—Guinea-pig serum preserved by Richardson's (1941) method; formolized sheep red-cells, and glycerinated anti-sheep cell serum ("Wellcome" brand Wassermann reagents) were obtained from Burroughs Wellcome and Co. The preserved complement was used within 1 hr. of dilution. Sheep cells were washed three times and adjusted volumetrically to a 6 per cent suspension in physiological saline. Suspensions were used on the day of preparation and on the following day only. When required, the cells were sensitized by incubation with an equal volume of an appropriate dilution of haemolytic antibody for 30 min. at 37° and were used immediately. When not in use, all reagents were stored at between 2° and 4°.

Principle of the method.—The concentrations of complement and haemolytic antiserum giving maximal haemolysis were kept constant in the presence of varying concentrations of

the anticomplementary test substance. The degree to which haemolysis was inhibited at each concentration of test substance was estimated from the difference between spectrophotometric determinations of the haemoglobin released from lysed cells in the presence and absence of the test substance. Comparison of the relative intensity of activity of different test substances was then possible by comparison of the respective concentrations necessary to produce a fixed degree of inhibition of haemolysis (50 per cent).

Procedure.—Triplicate serial dilutions in physiological saline in 0·25 ml. volumes were made from a stock 0·1 per cent (wt./vol.) solution of anticomplementary substance in physiological saline.

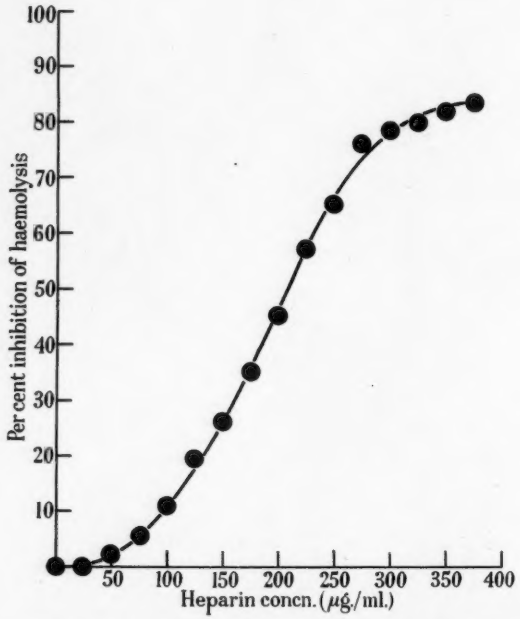

FIG. 1.—General nature of the relation between percentage inhibition of haemolysis and concentration of anticomplementary test substance (in the present instance, heparin).

To each tube, 0·25 ml. of a 1/50 dilution of complement, 0·25 ml. of sheep cells sensitized by an equal volume of 1/50 haemolytic antiserum and 0·25 ml. of saline were added. Two control tubes were included. In one the anticomplementary substance was replaced by an equal volume of saline, in the other, the complement was omitted, and replaced by saline. The results were taken as 100 per cent and zero haemolysis respectively.

The tubes were placed in a water bath at 37° for 30 min. and then in a refrigerator overnight at 4°. The following morning the tubes were centrifuged at 3000 r.p.m. for 10 min.: 0·4 ml. of the supernatant from each tube was then transferred to another tube containing 3·6 ml. of saline to give a suitable dilution of haemoglobin for the spectrophotometric measurement of optical density. Measurements were made at 4100 Å in a Unicam Spectrophotometer (Model S.P. 500) using 1 cm. glass cuvettes and employing the dilution from Control II (zero haemolysis) as the blank.

The difference between the optical density of each test dilution and that of control with 100 per cent haemolysis was expressed as a percentage of the control reading (*i.e.*, percentage inhibition of haemolysis produced at each concentration of the test substance).

RESULTS

It had previously been found that on plotting graphically the percentage inhibition of haemolysis against the concentration of anticomplementary test substance the curve expressing the character of the relation between these variables was sigmoid in shape (Fig. 1).

On the other hand, the percentage inhibition of haemolysis plotted on a probit scale against the logarithm of the concentration of the test substance

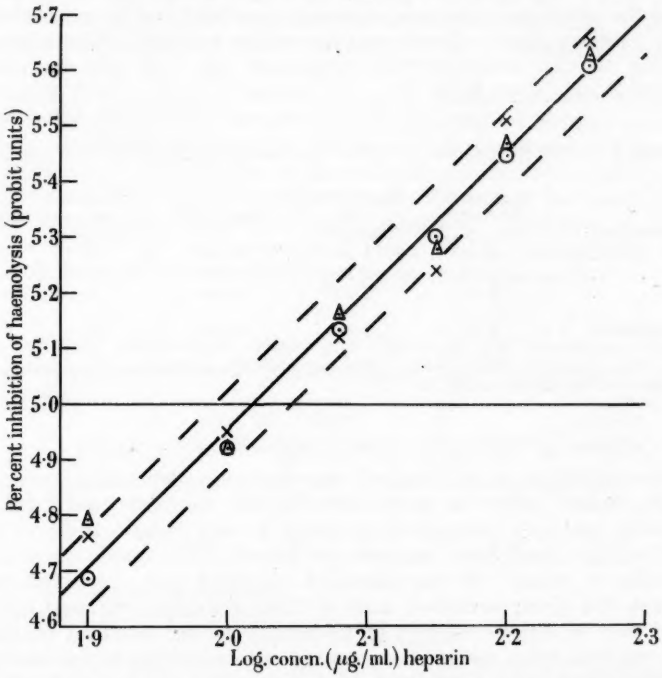

FIG. 2.—Relation as in Fig. 1 plotted as percentage inhibition of haemolysis (probit scale) versus log. of concentration (in μg./ml.) of heparin. Solid line: common regression for three runs. Interrupted line: 95 per cent confidence limits for common regression. x; △, ⊙: experimental points for runs 1, 2 and 3 respectively.

gave a relation which was approximately linear. Analysis of the probit/log. dose relation by the method of least squares showed a significant correlation coefficient (see Table I). Regression lines were calculated for each set of the triplicate experimental values. On comparing the regression coefficients and their intercepts on the y-axis by an analysis of variance, no significant differences were found between a common regression line and separate lines, nor between the intercepts. The common regression line was therefore regarded as an adequate fit. The validity of this procedure was checked in some instances by full probit analysis of the experimental values (three fits) without significant variation from the results obtained by the simpler procedure outlined.

Ninety-five per cent confidence limits were calculated for the common regression line in several similar experiments and it was found that the experimental points, with only an occasional divergence, fell within these limits (Fig. 2).

Since it was evident that in this, as in other biological experiments where the use of probit scales is appropriate, the greatest accuracy could be expected to be obtained around the probit value (5·0 probit units) corresponding to 50 per cent haemolysis, concentration ranges of anticomplementary substance giving values on either side of, but not too far from this value were chosen and the intercept of the calculated common regression line with the 50 per cent axis was taken as a primary index of anticomplementary activity. The concentration corresponding to this intercept was designated the " 50 per cent inhibitory concentration " (IC_{50}) (see Table I).

TABLE I.—*Determination of Anticomplementary Activity of Heparin*

Experiment	50 per cent* intercept (log. IC_{50})	Regression* coeff. (Slope)	Corr. coeff. (r)	Degrees of freedom	Significance of r
A	2·02	2·50	0·984	4	>0·001
B	2·01	2·38	0·990	4	>0·001
C	2·03	2·59	0·986	4	>0·001
Common regression	2·02	2·49	0·987	16	>0·001

* Derived from regression lines relating percentage inhibition of haemolysis (probit scale) and log. concentration of heparin as in Fig. 2.

Influence of Variability in the Components of the System

The reproducibility of the method was obviously dependent on the degree of variability which might be encountered in the reagents used. In the case of the heparin and the haemolytic antibody it was judged that no difficulty was likely to arise since these reagents are known to be stable on storage over many months or years. It was therefore assumed that, provided the same concentration of a given sample of each of these materials was used throughout, they would not be serious sources of variability in the results. On the other hand, the red-cells were received as weekly consignments and when made up in saline suspension showed evidence of deterioration on storage for a week or longer. Although an effort was made to eliminate variability from this source by standardizing the method of preparation of the suspension and by discarding cell suspensions more than 2 days old, it was judged advisable to investigate whether the " freshness " of the suspension within this age range affected the results and also whether there was inherent variability between different batches of sheep cells.

Complement preserved by Richardson's method is known to be stable for six months or longer if stored between 2° and 4°. However, since complement is a biological reagent of complex nature, consisting of at least four components which may be present in variable proportions, it was considered likely that variation might be encountered between batches of the preserved material. Moreover, since the preserved reagent is not universally available it was thought advisable also to investigate variability between batches of freshly obtained guinea-pig serum.

With these considerations in mind, a series of replicate experiments, each similar to the one described above, was designed in the form of a completely balanced three factor block to allow simultaneous evaluation of the possible influence upon the value of the IC_{50} and the regression coefficient (slope) obtained for the heparin sample as a result of using (1) 3 different batches of sheep cells, (2) each batch of cells as a suspension within 4 hr. of preparation and when 24 hr. old, and (3) two different batches of preserved complement. The haemolytic antibody concentration was kept constant throughout. This design also allowed evaluation of possible interactions between these variables. The influence of using different batches of fresh complement was investigated in a separate experiment.

The results (Table II) were submitted to an analysis of variance from which it was concluded (Table III) that the use of (1) different batches of sheep cells produced no significant variation in intercepts but variation of a low order of significance in slopes, (2) the ages of the cell suspensions at the two levels tested had no significant influence upon either slope or intercept, but that (3) different batches of complement exerted a highly significant influence on the values obtained for both slope and intercept. There was no statistically significant evidence of interaction between the variables in this experiment.

TABLE II.—*Values Obtained for Slopes and Intercepts with Constant Concentration of Haemolytic Antibody and Variation of the Other Components of the Haemolytic System*

		Log. IC_{50} values*			Slopes*		
		T_1	T_2	T_3	T_1	T_2	T_3
A_1	W_1	2·22	2·25	2·24	2·78	3·52	3·11
	W_2	2·17	2·29	2·18	3·07	3·28	3·63
A_2	W_1	2·05	1·98	2·02	2·33	2·32	2·75
	W_2	1·98	2·02	1·97	2·61	2·49	2·74

T_1, T_2, T_3: batch numbers of sheep-cells;
A_1, A_2: batch numbers of complement;
W_1: fresh cell suspension; W_2: 1-day-old cell suspension.
* Each result derived from the common regression of an experiment in triplicate.

TABLE III.—*Results of Analysis of Variance on Data Obtained in Table II*

Source of variance tested against residual variance	Degrees of freedom (n_1/n_2)	IC_{50}		Slope	
		Variance ratio	Significance	Variance ratio	Significance
Between cell suspensions	1 : 7	1·1	<0·20	0·9	<0·20
,, red cell batches	2 : 7	0·5	<0·20	4·7	0·05
,, complement batches	1 : 7	75·6	>0·001	41·0	>0·001

Even more marked variation was encountered when the heparin sample was titrated against five batches of fresh complement, each derived from a different guinea-pig. Values ranging between 1·85 and 2·15 were obtained for the log. concentrations corresponding to the 50 per cent intercept (IC_{50}) while the regression coefficients varied between 1·74 and 3·86 (Fig. 3).

Influence of haemolytic antibody concentration

From the foregoing experiments it is evident that reproducible results were obtained with a fixed concentration of haemolytic antibody. It has long been known that a direct relation exists between the concentration of haemolytic antibody present in a haemolytic system and the concentration of complement necessary to produce a given level of haemolysis (Osborne, 1937). Where an anticomplementary substance was introduced into the system it seemed reasonable

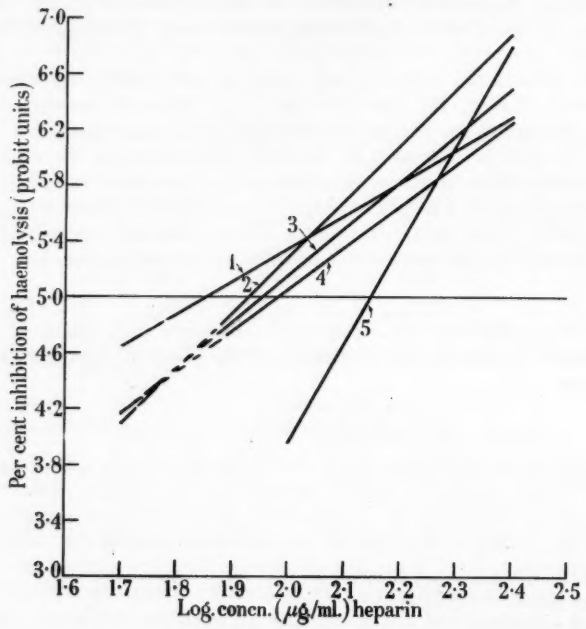

FIG. 3.—Variation of slope encountered in titrating a single sample of heparin against five different batches of complement. The experimental points have been omitted for clarity, the lines being the regressions for each titration.

to expect competition between this substance and the haemolytic antibody for interaction with the complement. For example, it seemed advisable to investigate whether, when haemolytic antibody was present in increasing concentrations, the corresponding concentration of complement left unbound by haemolytic antibody (and therefore free to interact with and become neutralized by the anticomplementary test substance) would be correspondingly altered. In this event the apparent anticomplementary activity of the test substance would also be affected. Experiments were devised to test this hypothesis as follows :

Relation between haemolytic antibody concentration and complement titre

A single batch of preserved complement was titrated against a single batch of sheep cells sensitized with haemolytic antibody at four different levels (*i.e.*,

dilutions of 1/50, 1/100, 1/150 and 1/200). After incubation, the percentage haemolysis in each series was estimated spectrophotometrically, as before. In this instance, the percentage haemolysis expressed in probit units was plotted against the reciprocal of the dilution of complement. A linear relation was obtained, allowing the amount of complement (HC_{50}) associated with the production of 50 per cent haemolysis in each series to be obtained from the intersection of the regression with the axis corresponding to 50 per cent (Table IV, column 2).

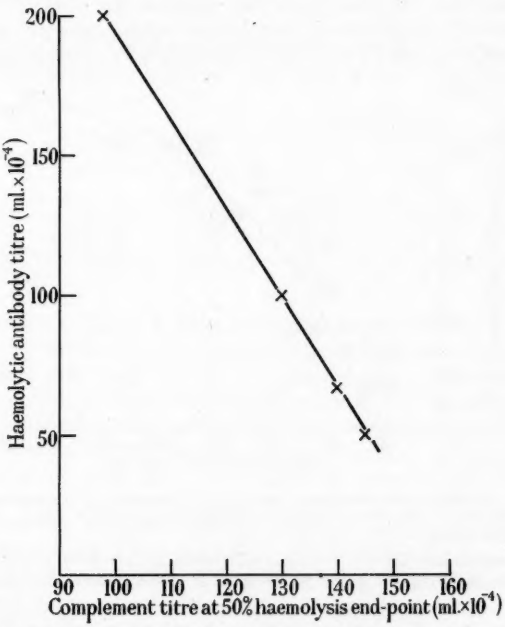

FIG. 4.—Relation between amounts of haemolytic antibody and of complement giving a 50 per cent haemolysis end-point.

TABLE IV.—*Relation between Haemolytic Antibody Concentration, Complement Titre for 50 per cent Haemolysis (HC_{50}) and Anticomplementary Activity (IC_{50}) of Heparin*

Haemolytic antibody concn. (ml. × 10^{-4})	Complement titre for 50% haemolysis (HC_{50}) (ml. × 10^{-4})	Log. IC_{50} heparin	Excess complement neutralized by heparin (ml. × 10^{-4})
200	97	2·03	103
100	130	1·80	70
66·7	140	1·71	60
50	145	1·68	55

On now plotting the amount of haemolytic antibody used to sensitize the cells in each series against the HC_{50} obtained from the same series, a linear relation was established (Fig. 4).

Relation between haemolytic antibody concentration and anticomplementary activity

The same sensitized cells that were used in the previous experiment were now used for another series of tests in which the concentrations of heparin were varied in each series. But, on this occasion, complement was added to each series at a constant dilution of 1/50. The IC_{50} of the heparin was estimated for each series as before (Table IV, column 3).

The amount of complement required to produce 50 per cent haemolysis at each level of haemolytic antibody was known from the previous experiment (Table IV, column 2). It was therefore possible to obtain by difference the additional amount of complement neutralized when heparin was added to the system as described in Table IV, by assuming that for any given level of haemolytic

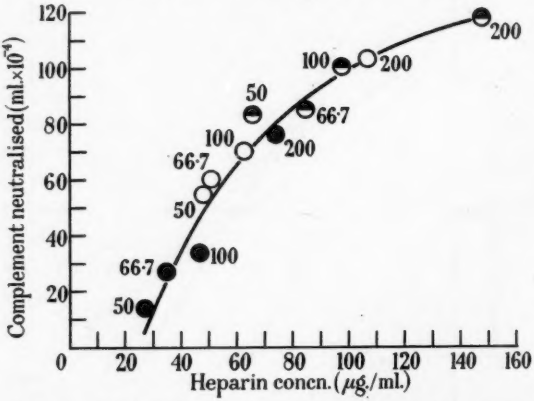

FIG. 5.—Relation between concentration (μg./ml.) of heparin and amount of complement neutralized at varying levels of haemolytic antibody and at different haemolytic end-points (25, 50 and 75 per cent).

● = 25 per cent haemolysis; ○ = 50 per cent haemolysis; ◓ = 75 per cent haemolysis. The figure beside each plotted point represents the concentration of haemolytic antibody (ml. × 10^{-4}).

antibody this was represented by the difference between the complement concentration estimated at this level of haemolytic antibody and the fixed concentration (200 × 10^{-4} ml.) of complement. These differences are shown in column 4 of Table IV. Similar calculations could be performed for other end points (*e.g.*, 25 and 75 per cent haemolysis).

On plotting the concentration of heparin against the amount of complement neutralized at each level of haemolytic antibody and at different haemolytic end points the relation illustrated in Fig. 5 was obtained.

From the results of the above experiments it was concluded that accurate definition of the activity of anticomplementary substances in terms of their IC_{50} values demanded careful standardization of the reagents of the haemolytic system and, ideally, the employment of an identical set of reagents throughout. Even where these criteria are fulfilled, it is evident from Fig. 5 and Table IV that the IC_{50} is a relative value dependent on the level of haemolytic antibody adopted.

The primary purpose of the present investigation was to devise a method allowing comparisons between a number of compounds in a series of experiments

expected to be sufficiently large to render it difficult to maintain exact standardization of the reagents throughout. A modification of the treatment of results was sought to make possible comparisons of experiments done with different batches of reagents, and especially, different batches of complement.

The two batches of fresh complement which had been found to give the widest variations of slope and position of the regression lines for heparin (Fig. 3) were used in an experiment in which the activities of two different dextran sulphates were compared with that of the heparin preparation by the method outlined previously.

TABLE V.—*Comparison of Anticomplementary Activity of Heparin and Two Dextran Sulphates Using Different Batches of Fresh Complement*

	Complement					
	Batch 1			Batch 2		
Compound	Log. IC_{50}	Slope	Activity (A.C.U.)	Log. IC_{50}	Slope	Activity (A.C.U.)
Heparin	1·85	1·74	*100*	2·25	3·86	*100*
Dextran sulphate GD	2·25	6·13	39·4	2·67	6·08	37·6
Dextran sulphate I/7	1·95	5·52	79·9	2·33	6·26	81·3

It can be seen from Table V that with the second batch of complement the IC_{50} values and the slopes of the regression lines were markedly different from those obtained with the first batch of complement. However, the ratios of the IC_{50} values for the various compounds relative to one another appeared to be similar for each complement batch. For greater ease of numerical expression, the IC_{50} value for heparin (S) relative to that for any given dextran sulphate (t) was expressed as a ratio S/t. The heparin sample was then assigned an arbitrary value of 100 " anticomplementary units " (A.C.U.) per mg. The anticomplementary activity of each dextran sulphate was then expressed in these units by simple derivation from the value of the ratio S/t (*e.g.*, if the ratio S/t for heparin and dextran sulphate X was $\frac{1}{2}$, the anticomplementary activity of dextran sulphate X was expressed as 50 A.C.U. per mg.).

It can be seen from Table V that the activities of the dextran sulphates relative to heparin, thus expressed, agreed within $\pm$ 2 per centw hen the results with the two batches of complement were compared.

It appeared, therefore, that although no absolute value for anticomplementary activity could be assigned to a particular compound it was justifiable to adopt an arbitrary reference preparation as a standard and to express the activities of other compounds relative to this standard regardless of the observed differences in slope and position of the regression lines with alteration of the batches of complement.

DISCUSSION

In the past, comparisons of anticomplementary activity between compounds have been made in terms of the concentrations of the compounds necessary to give *complete* inhibition of the haemolytic activity associated with a given volume of complement (Ecker and Pillemer, 1941 ; Gordon and Atkin, 1941 ; von Kaulla and Husemann, 1946 ; Mutsaars and Lison, 1948 ; Storti and Vaccari,

1956). But it will be noted that the curve relating inhibition of haemolysis to concentration of anticomplementary test substance (Fig. 1) is of similar sigmoid shape to that characteristic of the relation between haemolysis and complement concentration (*cf.* Fig. 5*a*, Kabat and Mayer, 1948) as might be expected. It has been pointed out by Kabat and Mayer that in such a situation the most sensitive index of activity is an end point in the region of 50 per cent haemolysis where the reaction is most sensitive to small changes in the amount of complement available.

With regard to the determination of the 50 per cent unit, the general design of the present method, although evolved independently, is similar in essence to a method described by Heidelberger, Jonsen, Waksman and Manski (1951) for the measurement of variation in haemolytic activity with variation in the components of complement. In agreement with these investigators, it is considered that designs incorporating the probit/log. dose relationship between percentage haemolysis (or, as in our case, inhibition of haemolysis) and the factor or factors influencing the amount of complement available, are simpler to apply, allow greater precision in measurement of slopes and intercepts, and are more soundly based than designs based on the use of the logistic function expressed in the von Krogh formula (Waksman, 1949), the use of the Angular Transformation as suggested by Fisher (quoted by Waksman, 1950) or the empirical formula proposed by Ponder (1948).

The analytical treatment applied to the data derived from the present design has allowed evaluation of the significance of variations between slopes and intercepts with variation of the conditions of the experiment in a manner which, to our knowledge, has not been reported previously. It was shown that using components of the haemolytic system, each from a single batch throughout, results of an acceptable degree of accuracy and reproducibility were obtained in replicate experiments. When the concentration of haemolytic antibody was kept constant, batch variations in the red cells had no influence on the IC_{50} value and relatively little influence on the slope whereas the use of different batches of complement markedly affected the values obtained for both slope and intercept.

In this connection it is noteworthy that Waksman (1950) found when making quantitative titrations of individual components of complement that, on measuring the haemolysis brought about by each of a series of mixtures of test components with increasing concentrations of a reagent lacking one component, the regression lines relating percentage haemolysis (in probit units) to log. dose also showed variation in slope and the values of the 50 per cent intercept. Waksman asserted that there was theoretical and experimental justification for the use of data determining 50 per cent haemolysis in the presence of moderate changes of slope of the regression line although rigorous statistical theory does not recognize lines of different slope as comparable.

While the present investigation supports the assertion that the 50 per cent value is a valid index of activity where the components of the haemolytic system are strictly standardized, and preferably, where comparisons are made using reagents from a single batch, it is clear that this value is a relative one, dependent on the level of haemolytic antibody chosen (Fig. 5, Table IV) as well as the batch of complement used.

The empirical observation that compounds showing differing degrees of anticomplementary activity maintain similar ratios of activity, relative to one

another, independent of variations between batches of complement, offers an alternative method of standardizing the technique for comparing the activities of such compounds.

SUMMARY

Measurement of the anticomplementary activity of heparin and related compounds has been carried out by a method allowing accurate determination of the concentration producing 50 per cent haemolysis (IC_{50}) in a standard haemolytic system. It was found that variation between batches of complement and in the concentration of the haemolytic antibody affected this value, but reproducible results were obtained, irrespective of these variables, when the activities of the test substances were expressed relative to that of a single heparin preparation arbitrarily chosen as a standard.

Our grateful thanks are due to Dr. C. R. Ricketts who supplied the dextran sulphate, Mr. M. Hall for analytical data on the heparin sample used, Dr. B. Haman for advice concerning the statistical design of the experiments and to Professor J. R. Squire for his interest, encouragement and helpful criticisms at all stages of this investigation. One of us (H. A. E.) is in receipt of a research grant from the United Birmingham Hospitals Endowment Fund.

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THE MOLECULAR CHARACTERISTICS DETERMINING THE ANTICOMPLEMENTARY ACTIVITY OF DEXTRAN SULPHATES

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IT has previously been reported that dextran sulphates resemble heparin in inhibiting anticomplementary activity and that when a series of dextran sulphates of varying molecular characteristics is examined, a considerable range of variation in the degree of this activity is encountered (Walton and Taylor, 1955).

Although it has long been known that many charged macromolecular materials are anticomplementary, comparisons of activity have been made, for the most part, between widely dissimilar compounds, often of incompletely defined chemical structure and by relatively insensitive methods for the detection of small differences in activity.

We describe here the application of a relatively precise method (described in the preceding paper) using an arbitrarily chosen heparin preparation as a standard, for quantitative comparisons of anticomplementary activity between the members of a series of compounds all derived from the same parent glucose polymer, dextran, but differing in a defined manner in molecular size and charge.

Since variation of these physico-chemical characteristics also influences *anticoagulant* activity, it has also been possible to investigate the relation between anticomplementary and anticoagulant activities in the same compounds.

MATERIALS

Dextran sulphate.—Sulphuric esters were prepared by the method described by Ricketts (1952) from dextrans varying in degree of polymerization from a pentasaccharide to a polymer containing about 6000 glucose units. The dextran sulphates varied in sulphur content from 1·92 per cent to 17·2 per cent.

Heparin.—The compound adopted as an arbitrary reference standard in the present investigation differed from that previously employed (Walton, Ellis and Taylor, 1957). Throughout the present investigation the preparation employed was "Pularin" (Evans) batch no. N10090. The anticoagulant potency of this compound was 90 I.U./mg. and it was found to contain 1·96 per cent nitrogen and 8·61 per cent sulphur; $(\alpha)_D^{20} = +42{\cdot}3°$

Haemolytic system.—Fresh guinea-pig serum diluted with physiological saline was used as the source of complement. Sheep's formolized red-cells, thrice washed and adjusted to a 3 per cent suspension in physiological saline, together with glycerinated anti-sheep cell serum (Burroughs Wellcome) formed the indicator system.

Method

The method outlined in the preceding paper was used to determine the concentrations of anticomplementary test substances producing 50 per cent inhibition of haemolysis (IC_{50}) under standardized conditions. It was shown that the IC_{50} value for any given test substance varies with the concentration of haemolytic antibody in the haemolytic system.

In the present investigation a convenient level of haemolytic antibody concentration was found to be 15 M.H.D. and this concentration was kept constant throughout the experiments to be described.

During the evolution of the method (preceding paper) the indicator system was prepared by sensitization of a given volume of a 6 per cent suspension of red cells by incubation with an equal volume of the chosen dilution of haemolytic antibody: 0·25 ml. of this mixture was then added to 0·25 ml. of complement, 0·25 ml. of anticomplementary test substance and 0·25 ml. of saline. In the present investigation, haemolytic antibody and 3 per cent red cell suspensions were added as separate 0·25 ml. aliquots to the system immediately before use. This slight variation in the technique produced no significant alteration in the results obtained with the method.

The previous investigation had also established that variation between batches of complement affected both the slope and the 50 per cent intercept of the regression line relating percentage inhibition of haemolysis (on a probit scale) and the log. concentration of a given test substance. But the intensities of activity of different test substances, relative to one substance arbitrarily chosen as a standard, remained constant. In the present investigation a heparin preparation was again used as a standard and arbitrarily assigned an activity of 100 anticomplementary units (A.C.U.). The activities of the various dextran sulphates in experiments using different batches of fresh complement were then expressed in the same units relative to this standard, as previously described.

Terminology.—In referring to the components of complement the terminology and symbols suggested by Pillemer and Ecker (1941) have been employed.

RESULTS

Comparison of the Anticomplementary Activity of Different Dextran Sulphates

The activity of four dextran sulphates was compared with that of the reference heparin preparation using one batch of complement. The parent polysaccharides from which the dextran sulphates were derived ranged in molecular size from a pentasaccharide to a compound containing about 1350 glucose units. The final compounds varied in sulphur content from 12·6 per cent to 17·1 per cent.

TABLE I.—*Comparison of Anticomplementary Activities of Various Dextran Sulphates Relative to Heparin Using One Batch of Complement*

Compound	Average number of glucose units	Sulphur content per cent	IC_{50}(μg.)	Slope	Activity (A.C.U.)
SS	5	15·1	48·98	3·47	35·9
I/4	20	17·2	37·30	3·30	47·1
E/2	140	12·6	1·64	11·07	1072·0
D/3	1350	15·4	1·29	14·75	1386·0
Heparin	? 52	8·61	17·58	3·87	*100·0*

It can be seen from Table I that the dextran sulphates derived from polymers composed of 5 and 20 glucose units showed respectively about one-third and one-half the activity of the standard heparin while the dextran sulphates derived from the polymers composed of 140 and 1350 glucose units were respectively about 10 and 13 times more active than the heparin.

It thus appears that molecular size influences anticomplementary activity. The molecular size of the heparin preparation used was not known. Most commercial heparin samples are generally acknowledged to be disperse in molecular size. Gronwall, Ingelman and Mosimann (1945) and Jensen, Snellman and Sylvén (1948) obtained estimates of 16,000–17,000 as averages for the distribution of

molecules in the preparations they examined. Marbet and Winterstein (1952) have pointed out that a molecular weight of this order suggests a molecule containing about 13 tetrasaccharide units (52 monosaccharide units) and this value has been accepted tentatively as applicable to the compound used in the present investigation (Table I).

From this experiment it was not possible to decide whether variation in sulphur content (*i.e.*, in the intensity of the charge conferred on the molecule by variation in the numbers of the strongly electro-negative SO_4 groups attached) might also contribute to the observed differences in anticomplementary activity. Accordingly, experiments were devised to allow separate investigation of the effect of variation in molecular size and sulphur content.

Effect of Variation in Molecular Size and Charge on Anticomplementary Activity

Molecular size.—The activity of three dextran sulphates of approximately equal percentage sulphur content (12·05–12·55 per cent) but of widely different molecular size was compared by reference against the standard heparin. It can be seen from Table II that increase in activity with increase of molecular size

TABLE II.—*Comparison of Anticomplementary Activities of Dextran Sulphates of Approximately Equal Sulphur Content but of Varying Molecular Size Using One Batch of Complement.*

Compound	Average number of glucose units	Sulphur content per cent	IC_{50}(μg.)	Slope	Activity (A.C.U.)
GD	20	12·55	33·90	4·87	37·7
GC	135	12·05	0·98	6·08	1300·0
GB	470	12·55	0·58	5·65	2197·0
Heparin	? 52	8·61	12·74	2·38	*100·0*

was confirmed. But, whereas increase in the degree of polymerization from 20 to 135 glucose units was accompanied by a thirty-five-fold increase in activity, increase in polymerization from 135 to 470 glucose units produced less than a two-fold increase in activity. The heparin preparation, although of different chemical structure, appeared to fall into place in the series in regard to the relation between molecular size and activity, provided it was assumed that the degree of polymerization adopted for the heparin was correct.

Sulphur content.—The relative activities of the members of two series of dextran sulphates were compared in further experiments. The first series comprised four compounds each derived from a dextran containing about 20 glucose units but varying in sulphur content from 1·92 per cent to 15·9 per cent. The second series consisted of two compounds, each derived from a dextran containing about 1350 glucose units, one compound containing 2·17 per cent sulphur and the other 15·4 per cent sulphur. Table III shows that in each series activity increased with increase of sulphur content.

The importance of the electro-negative charge imparted by the SO_4 groups was borne out by the further observation that the parent unsulphated dextran from either series, and even a crude dextran composed of several thousand glucose units, failed to show any trace of anticomplementary activity, even in concentrations as high as 20 per cent solutions.

TABLE III.—*Comparison of Anticomplementary Activities of Two Series of Dextran Sulphates Each of Equal Molecular Size but Varying in Sulphur Content Using Two Different Batches of Complement.*

Series	Compound (serial no.)	Sulphur content per cent	IC_{50} (μg/ml.)	Slope	Activity (A.C.U.)
(*a*) Large molecular size (1350 glucose units)	HA	2·17	0·63	7·41	1190·5
	D/3	15·40	0·56	13·78	1339·3
	Heparin	8·61	7·50	1·84	*100·0*
(*b*) Small molecular size (20 glucose units)	HB	1·92	58,550·0	8·60	0·00012
	CD	12·55	17·99	6·13	39·35
	BD	14·20	8·56	4·47	82·70
	BE	15·90	7·99	7·09	88·62
	Heparin	8·61	7·08	1·74	*100·0*

The Nature of the Interaction between Polysaccharide Sulphates and Complement

Evidence has been presented elsewhere (Taylor, 1956) that the inhibition of haemolysis in a haemolytic system by heparin and dextran sulphate is due to a direct action on complement and not on the red cells or haemolytic antibody. This is in keeping with the findings of Ecker and Gross (1929) for heparin and with those of Ecker and Pillemer (1941) for other polysaccharide sulphates.

Complementary activity is known to be an aggregate effect of several components. The anticomplementary effect of the compounds used in the present investigation appeared to be exerted on the heat-stable component of "endpiece". The main effect was thought to be exerted on C_4 but some effect on C_3 was not excluded (Taylor, 1956). Mutsaars and Lison (1948) found that starch and cellulose sulphates affected C_1, C_2 and C_3.

Relation between Anticomplementary and Anticoagulant Activity

The dextran sulphates showed considerable differences in anticomplementary activity (the difference between the least active compound, dextran sulphate HB, and the most active compound D/3, being over a million-fold). But the same compounds showed a very much smaller range of difference in anticoagulant activity (Table IV).

TABLE IV.—*Comparison of Anticoagulant and Anticomplementary Activities of Dextran Sulphates Relative to Heparin*

Compound (serial no.)	Average number of glucose units	Sulphur content per cent	Activity relative to heparin: Anticoagulant (International heparin units per mg.)	Activity relative to heparin: Anticomplementary (A.C.U. per mg.)
SS	5	15·10	9·3	35·9
HB	20	1·92	*Nil*	0·00012
I/4	20	17·20	18·0	47·1
E/2	140	12·60	11·75	1071·9
HA	1350	2·17	2	1190·5
D/3	1350	15·40	68·0	1386·0
Heparin	? 52	8·61	*100·0*	*100·0*

Moreover, while one actively anticomplementary dextran sulphate (compound HA) showed considerably greater activity than the reference heparin preparation, its anticoagulant activity relative to the same heparin preparation was almost *nil*.

DISCUSSION

It has long been known that the anticomplementary effect of charged macromolecular materials is qualitatively similar whether the charge is negative or positive (Ecker and Pillemer, 1941). It can also be inferred from previous work that anticomplementary activity is not specific to particular acidic or basic radicles.

For instance, compounds owing their electro-negative charges to sulphate or sulphonate radicles (heparin, sulphuric esters of other polysaccharides and synthetic sulphonated compounds like Liquoid, germanin and salvarsan) are highly active (Stuber and Lang, 1930; Klopstock, 1932; von Falkenhausen, 1931; Ecker and Pillemer, 1941). But a polymer owing its negative charge to another acidic radicle (hexametaphosphate) is also active though much less so than the foregoing compounds (Gordon and Atkin, 1941).

On the other hand, strongly basic (electro-positively charged) compounds such as protamine, diethylamine and various basic dyes also show anticomplementary activity (Wising, 1938; Pillemer, Seifter and Ecker, 1941; Mutsaars and Lison, 1948).

It is known that such macromolecular materials in solution behave as polyanionic colloids. For the dextran sulphates (Walton, 1952; Oncley, Walton and Cornwell, 1957) as for heparin and other polysaccharide sulphates (Bernfeld and Nisselbaum, 1956) it has been demonstrated that loose complexes are formed with the plasma globulins, which, for the present purpose, may also be regarded as hydrophilic colloids.

Theoretically, the intensity of the intermolecular forces governing colloid interactions is enhanced (*a*) when the concentration of one or both reactants is increased, (*b*) when the surface area of one or both reactants is increased, and (*c*) with increase in the number of, or in the intensity of, the charge upon their reactive groups (Anderson, 1946).

From this viewpoint, the results of the present experiments may be interpreted as follows:

Effect of concentration.—It was shown in the preceding paper that when the concentrations of the components of the haemolytic system were standardized, a direct relation could be demonstrated between the concentration of anticomplementary substance and the amount of complement neutralized. The relation was dependent on the concentration of haemolytic antibody present since when this was increased, the intensity of activity of the anticomplementary substance was correspondingly diminished.

It is known that sensitization of the red cell occurs by adsorption of the globulin molecule of the haemolytic antibody on the red cell surface (Heidelberger, Weil and Treffers, 1941; Haurowitz and Yenson, 1943). The sensitized red cells then react with other globulin components of the serum which together comprise complement. The present results agree with previous work in suggesting that anticomplementary substances intervene in this system by interference with the interaction between the antibody globulin absorbed on the cell surface and the globulin components of complement.

The diminution of anticomplementary effect with increase of haemolytic antibody may be explained as occurring by reversal of the competition between the charged polysaccharide and the red cell antibody complex for union with complement, the weaker interaction between complement and antibody being favoured by excess of antibody molecules.

Effect of surface area.—Dextran sulphates of large molecular size showed considerably greater activity than their analogues of smaller molecular size, weight for weight. Although hitherto the range of compounds used in this experiment has been considered solely in terms of the *numbers* of glucose units in the polymer, the spatial arrangement of these units would also affect the charged surface area available for interaction. It is known that the naturally occurring dextran molecule is highly branched (Ricketts, 1951) and that during degradation by hydrolysis the degree of branching decreases. The present series probably differed in molecular *shape*, therefore, as well as size, the higher molecular weight fractions being highly branched and offering a large surface area, the lower molecular weight fractions being less branched or filiform and offering a smaller surface area for interaction with the globulin components of complement. von Kaulla and Husemann (1946) have noted differences between the anticomplementary activity of other polysaccharide sulphates differing in molecular size and shape. Gordon and Walker (1945) concluded that the anticomplementary activity of colloidal dyes related to Congo red was more dependent on alterations in molecular size than on variation of specific groupings within the dye molecule.

Effect of charge.—Neutral (unsulphated) dextrans, regardless of molecular size, were found to be inactive. Increase in the number of sulphate groups added at any given level of molecular size was attended by an increase in activity. However increase of charge produced different degrees of effect in the small and the large molecular weight series of compounds. For example, it can be seen from Table III that in the large molecular weight series, increase of sulphur content from 2·17 per cent to 15·4 per cent produced only 12 per cent increase in activity while on the other hand, in the compounds composed of 20 glucose units or less, increase of sulphur content over approximately the same range produced 700,000-fold increase in activity.

Although molecular size and shape have been considered separately from charge above, it should be noted that variation in molecular charge, in itself, probably brings about variation in molecular configuration, *i.e.*, the addition of strongly charged groups to a polymer composed of a given number of component units is likely to produce unfolding of the molecule by virtue of the mutually repulsive forces exerted by the charges bringing about elongation of the polymeric chains.

Relation between anticoagulant and anticomplementary activities.—It was suggested by Fuchs and his co-workers (for references see Quick, 1935) that prothrombin is identical with the midpiece of complement. On this basis efforts have been made in the past to relate anticoagulant and anticomplementary activities. But it was shown by Quick that the conversion of prothrombin to thrombin during the clotting process is not accompanied by decrease of complement and that other points of difference are detectable between the behaviour of complement and prothrombin, one being the quantitative difference in the intensity with which heparin reacts with each. The present investigation confirms the dissociation between these two forms of biological activity for the

dextran sulphates also, in that it has been shown by Forwell and Ingram (1956) that dextran sulphate has little or no antiprothrombic effect whereas the present results show a strong anticomplementary effect.

SUMMARY

It has been found that concentration, molecular size and sulphur content all influenced the anticomplementary effects of dextran sulphates relative to that of an arbitrary reference preparation of heparin.

Dextran sulphates of larger molecular size than heparin were more active while dextran sulphates of smaller molecular size were less active than heparin.

Unsulphated dextran, regardless of molecular size, was inactive. The addition of sulphate groups produced greater differences in activity between dextran sulphates of small molecular size than among compounds of large molecular size.

It is suggested that these results are explicable on the basis that the over-all factor determining the anticomplementary activity of dextran sulphates is the charged surface area available for interaction with one or more of the globulin components of complement.

Quantitative comparisons between the anticomplementary and anticoagulant activities of the dextran sulphates suggest that these forms of biological activity are unrelated in their mechanism.

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STUDIES ON THE PATHOGENICITY OF *STREPTOCOCCUS PYOGENES*. IV. THE RELATION BETWEEN THE CAPACITY TO INDUCE FATAL RESPIRATORY INFECTIONS IN MICE AND EPIDEMIC RESPIRATORY DISEASES IN MAN

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IT has been shown by Coburn, Evans and Nathan (1954*a*, *b*) that with aerosol spraying certain strains of group A haemolytic streptococci have the capacity to induce fatal respiratory tract infections in the Swiss albino mouse whereas others do not. This capacity of the strain to induce fatal infection of the respiratory tract, as evidenced by bacteraemia, has been termed *infectivity*. This is independent of *virulence* as determined by the intraperitoneal injection of suspensions of the same culture. More recent studies by Slade, Coburn and Simich (1955) with " radioactively tagged " cocci have indicated that the critical factor in infectivity of an " epidemic " strain, as determined by the air-borne induction of fatal respiratory disease, is related neither to the number of micro-organisms in the air nor to the number inhaled by the mouse.

Previous studies have been made in most instances with suspensions of streptococci grown from lyophilized cultures stored for months or even years. The history of these strains, as they affected man, was known. Some had induced epidemics of streptococcal nasopharyngitis, with or without scarlatina. Irrespective of serological type and mouse virulence, most strains had caused only sporadic streptococcal disease, *e.g.*, tonsillitis, pharyngitis, and peritonsillar abscess, in which cross-infections rarely occurred. None of these studies provided information on the infectivity of group A streptococci which had not been lyophilized. Hence, it was not known whether " non-epidemic " streptococcal strains freshly isolated from the human nasopharynx, but not associated with cross-infections, can induce fatal respiratory tract disease in the mouse.

The present study was undertaken with two objectives ; namely, to determine whether : (*a*) the previous observations made on the lyophilized cultures could be confirmed with freshly isolated strains of group A haemolytic streptococcus ; and (*b*) a streptococcal outbreak could be foretold by aerosol spraying of mice before epidemic spread had developed. This report presents the epidemiologic and experimental findings on these points made during the 2-year period, September 1954–August 1956.

MATERIALS AND METHODS

Most of the materials and methods used in this study, including the air-tight spraying chamber, culture medium, bacterial suspensions, mouse-adapted PR8 influenza virus strains, anaesthesia, autopsy examination and serological identification of haemolytic streptococci recovered at autopsy, were identical with those described previously (Coburn *et al.*, 1954*a*, *b*).

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Throat cultures

A throat culture was made on each mouse upon removal from the chamber following 3 days' exposure to the aerosol spray of streptococci. Subsequent throat cultures were made at intervals of 2 to 3 days and just prior to autopsy either following fatal infection or sacrifice at conclusion of the 10-day incubation period. Of the haemolytic streptococci recovered, an appropriate sample was tested to make certain that the strain was serologically group A and of the type to which the mouse was exposed.

Handling of Swiss albino mice subsequent to aerosol inoculation

Following removal from the chambers, each mouse was kept in the same individual compartment of the 6-mouse wire cage in which it had been housed when exposed to the aerosol spray. This was done to prevent cannibalization of mice dying at night.

Bacterial strains

With the exception of the control observations, streptococcal cultures used in this study were freshly isolated from the throats of young male adults diagnosed as having acute nasopharyngitis. Strains N19 and 070596 (Coburn *et al.*, 1954*a*, *b*) were used throughout this study as a positive control and negative control respectively. In a few instances strains isolated from sporadic streptococcal infections were stored for several weeks on sheep blood agar slants at 5° before being tested. In most instances, however, a colony was picked directly from the original 24-hr. throat swab culture on a blood agar plate. Single colony isolates were transferred to a tube of broth, tested for purity after 24 hr. incubation, and serologically typed. Another sub-culture was made into a flask of broth whence after 18–24 hr. aliquots were used to make bacterial suspensions for the aerosol inoculation spray.

Definitions

Infectivity.—As previously defined (Coburn *et al.*, 1954*a*) infectivity is " The capacity of a bacterium when inhaled by the mouse, in either droplets or dust particles, to multiply in the respiratory tract, cause cervical lymphadenitis, pneumonia and death associated with a positive blood culture." Throughout this work infectivity connotes not merely the ability of the bacterium to establish itself in the throat flora but also to cause death with bacteraemia.

Carrier state.—All mice having throat cultures positive for the streptococcal strain to which they had been exposed were considered to be in the carrier state. Some of these progressed to fatal streptococcal disease, whereas others survived. The duration of positive throat cultures varied from one day to weeks.

Benign carriers.—Mice with positive throat cultures in which the challenging group A streptococcal strain failed to invade cervical lymph nodes, lungs and blood stream were classified as benign carriers. In these mice the haemolytic streptococcus often became predominant in the throat flora throughout the period of observation. Nevertheless, these mice appeared to remain in good health.

EXPERIMENTAL

All sprayings were made in air-tight chambers on 12 mice in individually wired compartments. All calculations were made on either 6 or 12 mice.

Benign carriers.—Over a period of years in the development of the aerosol infectivity method, it had been observed that the infectivity of strain N19 was first manifested by positive throat cultures and followed in a few days by death. During the summer months of 1955, this strain (which had been lyophilized during winter experiments) was inoculated into broth directly from the dried state. When sprayed in the chamber, it infected the throat of exposed mice as shown by throat culture, but did not cause death. Under summer environmental conditions of the laboratory the mice thus appeared to escape fatal infection while still harbouring the N19 strain in the throat. This is shown in Fig. 1.

Furthermore, it was found that during the winter months of 1956, when strain N19 did induce mouse mortality rates of 30–60 per cent, many other sporadic

streptococcal types which had been associated with streptococcal infections caused persistently positive throat cultures without causing death. In brief, under certain conditions both infective and non-infective strains survived and multiplied in the naso-pharynx without causing death. This state is classified as benign carrier.

Moreover, irrespective of the strain of streptococcus tested a pre-conditioning of mice with a sublethal dose of PR8 influenza A virus pre-disposed to the benign carrier state. This was evidenced by higher percentages of positive

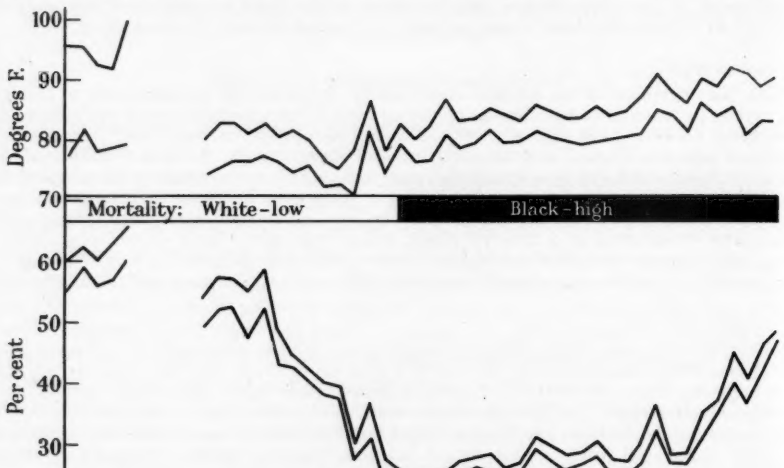

FIG. 1.—Seasonal variations in infectivity of a lyophilized laboratory strain, N19, in relation to the laboratory temperature and humidity. The break in the curve coincides with the summer holiday in which no tests were made. Throughout the experiments all mice were kept in the laboratory where the temperature and humidity fluctuated with the seasonal changes. Low mortality represents streptococcal death rates of 0–8 per cent; high mortality 33–83 per cent.

Upper graph—weekly averages of daily max. and min. temp.
Lower graph—relative humidity.

throat cultures in the pre-conditioned group than in the normal mice. Since all 12 mice in a compartment received the same exposure to an aerosol spray containing group A haemolytic streptococcus (Slade *et al.*, 1955), a variation in bacterial dose could not account for this difference.

Limitations of aerosol spraying technique.—Variations in mortality rates occur between compartments sprayed with aliquots of bacterial suspension in identical manner. For example, when 6 separate compartments containing 12 mice each are sprayed simultaneously with an infective strain, the aerosol spray within one compartment may fail to induce infection among normal mice. This is shown in Table I. Here it is seen that in compartment *d*, the 6 normal mice failed to develop positive throat cultures and none died of streptococcal disease.

Nevertheless, 4 out of the 6 mice which had received a preliminary sublethal dose of influenza A PR8 virus developed positive throat cultures and died with group A haemolytic streptococcal bacteraemia. One week later, however, when the experiment was repeated, compartment *d* gave the highest incidence of positive throat cultures and streptococcal deaths in the normal mice. The reason for these variations is unknown.

TABLE I.—*Variations in Infectivity with Aliquots of a Culture of N*19

	Normal mice		PR8 mice	
Compartment	Positive throat cultures	Strep. mortality	Positive throat cultures	Strep. mortality
a	1/6	1/6	4/6	4/6
b	1/6	1/6	2/6	2/6
c	1/6	1/6	4/6	4/6
d	0/6	0/6	4/6	4/6
e	2/6	2/6	3/6	3/6
	2/6	2/6	3/6	3/6
Average	1·2/6	1·2/6	3·3/6	3·3/6

Epidemiological Observations

The streptococcal cultures tested in this study were obtained from the throats of recruits at a U.S. Naval Training Center during a 2-year period. There were 3000 young men in training for a period of 10 weeks. Weekly throat culture surveys were made on approximately 7 per cent of each training regiment and cultures were obtained from persons reporting to Sick Bay with upper respiratory tract symptoms. All throat swabs were collected on dry swabs and streaked on sheep blood agar plates within 2 hr. after collection. After approximately 24 hr. incubation at 37°, β-haemolytic streptococcal colonies were sub-cultivated for pure culture study and lyophilized. During the 2-year period approximately 40,000 throat cultures were examined. Organisms of several different serological types were obtained from sporadic streptococcal infections. However, during each epidemic, practically all group A streptococci recovered were of a single serological type.

The epidemic beginning in January 1955 was temporarily checked with a course of penicillin prophylaxis. The programme consisted of 250,000 units of buffered penicillin G administered orally twice daily for 10 days. However, the epidemic reappeared in about 6 weeks and another similar course of penicillin prophylaxis was required. During this 4-month period there were approximately 3000 streptococcal infections in the recruit population. The epidemic type 19 organism disappeared from the throat flora during the summer of 1955. Nevertheless, following a fall outbreak of mild upper respiratory tract infections, which was probably of viral origin, type 19 reappeared sporadically in the throat flora of recruits. By mid-January 1956, the incidence of streptococcal throat infections rose sharply. At this time almost all strains recovered were again type 19. This incipient streptococcal epidemic was controlled by treating recruits with 250,000 units of oral penicillin twice daily for 10 days followed by 250,000 units daily for another 30 days. During this period and for an additional 6 weeks, incoming recruits were given a single injection of 600,000 units of benzathine penicillin shortly after arrival at the Training Center. For the second outbreak,

the peak incidence was 8/1000/wk. during the third week of January 1956. Associated with the first epidemic there occurred 81 cases of rheumatic fever and with the second epidemic there were 33. These observations are summarized in Fig. 2.

Bacteriological Findings

Each of these epidemic outbreaks was associated exclusively with a group A type 19 streptococcus. Prior to the first epidemic of 1955, other organisms were isolated from sporadic throat infections occurring among the recruits.

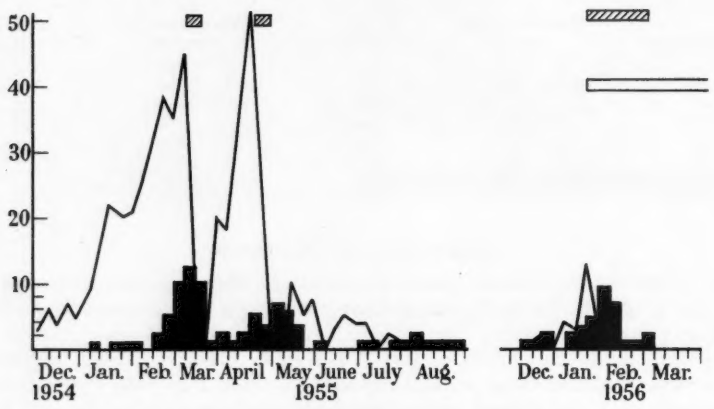

FIG. 2.—Outbreak of infection due to *Str. pyogenes*, type 19, in navy recruits at Great Lakes, Ill., 1954–56.

Throat cultures per cent containing Group A *Str. pyogenes*.

Cases of rheumatic fever per week.

Oral penicillin.

Benzathine penicillin injection on arrival.

These included types 1, 5, 8, 11, 12, 18, 19, 28 and numerous untypable group A streptococci. Prior to and following the streptococcal outbreak of 1956, types 1, 3, 5, 12, 18 and untypable group A strains were obtained from sporadic throat infections.

Results in Mice

Numerous freshly isolated type 19 streptococci were, at different stages in the epidemic, tested for infectivity for both PR8-conditioned and normal mice. In most instances each type 19 organism, picked at random, showed a high degree of infectivity. It killed normal mice, as well as those with mild influenza. Variations in infectivity of type 19 strains, 1954–56, are shown in Table II.

Identical measures were used for spraying representative strains of all other serological types of group A haemolytic streptococcus associated with acute

TABLE II.—*Differences in Infectivity of Type* 19 *during Various Stages of the Epidemic Process*, 1954–56

Period	Clinical state	PR8-infected mice: Positive throat cultures (per cent)	PR8-infected mice: Strep. mortality (per cent)	Normal mice: Positive throat cultures (per cent)	Normal mice: Strep. mortality (per cent)
December 1954–January 1955	Onset; low streptococcal morbidity rate	50	33	16	0
January–March 1955	Progressive rise in streptococcal morbidity rate	75	75	58	58
March 3, 1955	Peak of epidemic	85	73	91	67
March–April 1955		Penicillin.			
April–July 1955	Sporadic infections	50	8	50	4
August–November 1955		No type 19 organisms recovered.			
December 1955–January 1956	Onset of streptococcal morbidity rate rise	66	53	57	15
January 1956	Progressive rise in streptococcal morbidity rate	50	36	17	9
January–February 1956		Penicillin.			
February–March 1956	Sporadic infections (some in penicillin-sensitive persons)	50	34	4	2

TABLE III.—*Lack of Infectivity among Strains of* Streptococcus pyogenes *which caused Respiratory Diseases but were not Associated with Epidemics in Man*

Date of isolation	Serological type	Streptococcal mortality in mice: Normal mice	Streptococcal mortality in mice: PR8 mice
October 1954 to January 1955	1	1/12	1/12
	5	0/12	0/12
	8	0/12	0/12
	11	0/12	0/12
	12	0/12	0/12
	18	0/12	0/12
	19	0/12	0/12
	28	0/12	0/12
	Untypable strain	0/12	0/12
	„ „	0/12	0/12
	„ „	0/12	0/12
November to December 1955	5	0/6	1/6
	1	0/6	1/6
	12	0/6	1/6
	3	0/6	1/6
	18	0/6	0/6
	5 (T)	0/6	0/6
	3 (T)	0/6	0/6
	Untypable strain	0/6	0/6
January to March 1956	1	0/6	0/6
	1	0/6	0/6
	5	0/6	0/6
	5	0/6	0/6
	5	0/6	0/6
	Untypable strain	0/6	1/6
	„ „	0/6	1/6
Total	Twenty-six strains	1/222	7/222

infections during this period of study. All strains were tested during the cold weather months when the normal mouse mortality from the positive control, strain N19, varied between 30 and 70 per cent. Strains from sporadic infections were tested in parallel with the epidemic type 19 cultures. The composite findings on organisms which failed to induce an epidemic are shown in Table III. Some of these strains may have caused several cross-infections. Nevertheless, although the environmental conditions were conducive, none of them spread to more than a few contacts before either disappearing or reappearing only occasionally in a sporadic throat infection.

It is seen in Table III that 26 different strains of group A streptococci from sporadic infections were tested on 444 mice. Among the 222 normal mice there was only one streptococcal death, due to a type 1 organism. Altogether, there were 7 deaths among the 222 mice which had been inoculated with influenza A, PR8 virus. However, no bacterial strain caused more than one death among the mice exposed. These 26 different strains, freshly isolated from the throat of persons with acute pharyngeal infections, showed either no infectivity or minimal infectivity in the mice.

In contrast to these observations, the type 19 organisms obtained during epidemic periods produced a high incidence of streptococcal deaths. For example, in January 1955, type 19 cultures caused an average mortality of 58 per cent. The difference in mortality rates between normal and PR8-infected mice was not significant. At the height of the epidemic in March 1955, on the day prior to the institution of penicillin prophylaxis, type 19 appeared most infective. A nasal culture, picked at random, containing large numbers of mucoid colonies (strain 127991) caused a 71 per cent mortality in normal mice (17 of 24 animals). This organism was the most infective recovered during the 2-year period of study.

The 1955 epidemic was well advanced when the laboratory studies showed that type 19 was the only highly infective organism in the throat flora of the Navy recruits in training. Plans were made to determine whether it is possible to detect in advance the appearance of an epidemic strain in the throat flora. During October 1955, a battery of 6 air-tight compartments was arranged so that at least 6, and perhaps 12, cultures could be tested weekly. The decision was made to spray a culture of each type recovered from a throat infection on 12 mice, 6 of which had received PR8 influenza virus. Table III shows that organisms other than type 19, recovered during the fall and early winter months, caused little or no mortality in normal mice. At this time, type 19 had apparently vanished from the environment.

However, in December 1955, when type 19 was again recovered from the throat flora, associated with acute streptococcal infections, it induced a mouse mortality comparable to that observed in January 1955. The only difference observed was that in these infections, which followed an outbreak of " colds ", the type 19 cultures were significantly more infective in mice with influenza than in normal mice. The variations are shown in Table II.

The epidemic of January 1956 was interrupted in the early phase by antibiotic prophylaxis. At this time numerous streptococcal strains were tested. No increase in infectivity of type 19 was demonstrated during this 3-week period. However, there appeared to be definitely less infectivity among the type 19 organisms recovered from the few sporadic infections which occurred following

the effective suppression of both epidemics with penicillin. Whether these few cultures were the residua of the epidemic type 19 strain (or strains), or whether they were introduced into the environment by new recruits, is unknown. In March and April 1956, environmental conditions were still favourable to a streptococcal outbreak. None occurred. The type 19 present in the throat flora induced in normal mice a mortality rate of only 2 per cent (1 of 48). This was shown in Table II.

It was shown in Table I that the spraying technique has limitations. Even under apparently identical conditions of testing, one aliquot of an N19 suspension failed to cause streptococcal deaths among normal mice. For reasons unknown, these animals failed to develop positive throat cultures. Similarly, in the early phase of an epidemic, differences are noted between cultures. These may be due either to strain variations or to the limitations of the methods employed. Table IV illustrates this. It shows the results from 8 throat cultures selected at random. All of these infections had occurred within 18 hr. at the beginning of the epidemic and were proved serologically to be type 19. They produced in mice inoculated with influenza virus a high incidence of positive throat cultures, 16–83 per cent, and a high incidence of streptococcal deaths, 16–83 per cent. In contrast to type 19 strains recovered at the peak of the 1955 epidemic, these organisms produced in normal mice a relatively low incidence of positive throat cultures, 0–33 per cent, and an even lower incidence of streptococcal deaths, 0–16 per cent. It is not known whether the apparent failure of some of these organisms (shown in Table IV) to colonize the throat is due to the organism itself, to the mice or to some limitations of the spraying method.

TABLE IV.—*Differences in Infectivity of Strains Randomly Selected at Onset of Epidemic from Nine Patients with Naso-pharyngitis*

Date of isolation in 1956	Number of culture isolated from throat	PR8 influenza A-infected mice		Normal mice	
		% positive throat cultures	Strep. mortality	% positive throat cultures	Strep. mortality
January 17	1942	33	33	0	0
,, 17	1987	33	33	16	16
,, 17	2138	83	83	16	16
,, 17	2144	50	16	33	0
,, 17	2152	66	66	16	16
,, 18	2515	50	33	33	0
,, 18	2540	33	33	0	0
,, 18	2710	16	16	0	0

In brief, from the results of laboratory findings, it was possible to point out in December 1955 that a potentially epidemic strain of type 19 was present in the throat flora at the Great Lakes Naval Training Center. The rise in the streptococcal morbidity rates in subsequent weeks clearly showed that an epidemic was beginning. The administration of oral and parenteral penicillin to practically all recruits suppressed the epidemic and prevented the acquisition of knowledge on the intensity and duration of the streptococcal outbreak. Nevertheless, one fact is certain; initiation of the epidemic was due to a single serological type of group A streptococcus. In January 1956 all throat infections were caused by type 19.

Environmental Factors

It has long been recognized that there is a striking seasonal influence on the incidence of streptococcal infections of the upper respiratory tract. Whereas in Europe the peak incidence of scarlet fever customarily occurs in the last two months of the year, the peak is not reached in American cities (for example, New York and Chicago) until February or March. In these two cities there is customarily a precipitous drop in streptococcal nasopharyngitis (including scarlet fever) with the onset of summer. In the months of August to October, scarlet fever and other manifestations of streptococcal throat infections are uncommon and an air-borne epidemic (excluding the high altitudes in northern portions of the Rocky Mountains) is a rarity in the United States. In contrast, during these very months which provide winter to cities of the South Temperate Zone, scarlet fever reaches its peak incidence.

There seems to be a similar seasonal influence in Chicago on mouse susceptibility to highly infective strains. Each summer, over a period of 6 years, it has been observed that a laboratory strain (N19) was unable to induce respiratory infections among the mice. The onset of this period varied from May to August and its duration varied from 3 to 5 months. For example, May 1955 brought unseasonably warm weather to the Chicago area. Laboratory windows were opened and steam heat for the laboratory building was turned off. With this change over to summer weather in the environment of the laboratory, the infectivity of N19, the positive control strain (lyophilized culture) disappeared. The mortality rate for mice exposed to the aerosol spray of strain N19 fell precipitously from 60 per cent to zero. This was true for cultures lyophilized at different times during the winter months. Likewise, the mouse mortality rate among mice exposed to the highly infective type 19 Great Lakes epidemic strain 127991 (referred to above) fell from 70–16 per cent.

All technical procedures, so far as is known, and the lyophilized cultures tested during the summer months, were identical with those used during the winter months. Furthermore, so far as could be determined, there was no difference in the raising and feeding of mice. That the dispersion of the aerosol sprays was effective was shown by the fact that the mice did develop positive throat cultures during the summer months. For example, in one experiment during the warm weather of 1955, all 12 mice challenged with strain N19 had positive throat cultures one week after removal from their 3-day exposure in the air-tight chamber. Nevertheless, in contrast to the high mortality rate of the cold weather months, not a single mouse died. This was almost equally true for mice exposed to strain 127991. During the cold weather all animals with a positive throat culture died of streptococcal bacteraemia; whereas at the end of May, 5 of 7 animals survived. Later in the summer this strain caused no deaths even though it became established in the throat flora of most of the animals challenged. This state persisted through October 1955. In November, however, both strains N19 and 127991 again caused streptococcal mortality as well as positive throat cultures. By December the capacity of these two strains (the same lyophilized cultures) to induce a high mortality rate in the mice was again confirmed.

In brief, the rôle of environment in mouse susceptibility to respiratory tract infection with group A streptococcus is unknown. Nevertheless it appears that some factors associated with season exert an influence. One factor recorded in the climate of the laboratory was the humidity-temperature status. This is shown

in Fig. 2. During a period when the humidity within the laboratory was extremely low, mouse infectivity was high. Whether this is coincidental or may be a significant factor in the environmental influence on mouse susceptibility under experimental conditions remains to be determined.

DISCUSSION

Previous studies on infectivity of group A haemolytic streptococcus were made with lyophilized cultures. The present studies were made with freshly isolated cultures during an epidemic (1954–55) and just prior to the onset of an epidemic (1955–56). Representative cultures of streptococci from all nasopharyngeal infections in a U.S. Navy Recruit Training population were tested for mouse infectivity. The laboratory method employed, even with its limitations, appeared useful in screening freshly isolated bacteria for potential infectivity in man.

Throughout this period of observation the streptococcal outbreaks were associated entirely with type 19. Even after one or more courses of penicillin prophylaxis this type was not eradicated from the Training Center. Others have observed that immediately following penicillin prophylaxis an epidemic type 19 organism disappeared and was replaced by another type. For example, prior to this study Seal, Mogabgab, Friou and Banta (1954) reported :

> " The result of prophylaxis with the 500,000 unit daily dose in the fourth regiment was dramatic. Type 19, the most prevalent type before prophylaxis, was markedly reduced by the first course and never regained its prevalence in the six-week interval between courses. With the exception of Type 3 streptococci, the effect on the other prevalent types was equally marked. Type 12 streptococci were virtually eradicated. Type 3 streptococci were not prevalent before prophylaxis. In the interval between the first and second courses of prophylaxis this strain increased in prevalence and at the time the second course was begun was the most prevalent type. It persisted through the second course of prophylaxis."

The type 19 organisms recovered from our 1954–55 epidemic, which seemed to occur independent of an outbreak of virus infections, appeared similar to the organism recovered from the 1955–56 epidemic which followed an outbreak of " colds ". The character of the infections was the same as was the incidence and manifestations of an important sequela, rheumatic fever. However, there was one difference noted in the laboratory. The 1955–56 strains regularly caused a higher mortality in mice inoculated with PR8 influenza virus. It is not known whether this difference is real or only an indication that the organisms which were recovered in the early phase of an epidemic had not yet reached a degree of infectivity sufficient to cause a high mortality rate in normal mice. It was also not possible to determine, because of the variations in mortality rates which occur with the spraying technique, whether organisms of a single serological type giving rise to an epidemic have the same degree of infectivity.

One of the most striking limitations in the use of the nebulization method in Chicago seems associated with summer. During hot humid weather the capacity of the bacterium to cause fatal disease in the mouse has temporarily disappeared each summer. An understanding of the climatic factors which presumably

protect the mouse and thereby prevent fatal disease after challenge with a highly infective strain of haemolytic streptococcus, may reasonably be expected to provide information useful in the control of human streptococcal infections of the upper respiratory tract.

No attempt has yet been made to do exhaustive constitutional and metabolic studies on the differences between an infective group A strain (*e.g.* N19) and a non-infective strain (*e.g.*, 070594) of the same serological type. Furthermore, no attempt has yet been made to modify infectivity by varying the constituents of the culture medium. Observations on certain constituents of strain N19 used in this study have been made by Hess and Slade (1955). They compared N19 with three other strains of type 19 and pointed out that " The electrophoretic patterns could be distinguished on a quantitative basis, particularly with respect to components 5, 6, 7, 8 and 9 ".

Preliminary work has been done on the relation between one streptococcal constituent and the metabolism of the micro-organism (Slade, H. D., personal communication). In one pair of laboratory strains a difference in glucose metabolism was observed. Two strains of the same serological type were tested. One possessed no M protein, as judged by lack of precipitin formation with antiserum ; whereas the other gave the customary positive precipitin reaction. There was a difference between the two strains as to the rate at which the whole cells metabolized glucose. The cells containing M protein were about 4 times more active than those not containing the protein. This finding indicates that under comparable conditions the cells containing the M protein would be able to obtain energy from the dissimilation of glucose in considerably less time than the cells which apparently contained no M protein. This may be due to either a quantitative difference in the amount of enzyme in each cell and/or a difference in the rate of penetration of the sugar to the enzyme sites within each cell. The rapid acquisition of energy requirements by group A streptococci in the naso-pharynx and the production of substances required for overcoming the bacteriostatic effect of lymph and blood may well determine the capacity of haemolytic streptococcus to induce fatal respiratory tract infections in mice and epidemics in man. The acquisition of this basic information remains a key problem in understanding the epidemiology of *Streptococcus pyogenes*.

In conclusion, the problem is how does group A haemolytic streptococcus induce air-borne upper respiratory tract infections. This capacity of the micro-organism to cause disease in man and mouse seems to be dependent on both (*a*) some extrinsic qualities of the environment, which are probably physical ; (*b*) some intrinsic qualities of the bacterium, which are probably metabolic. One or both of these factors may be intensified by concomitant viral infection ; for example, PR8 influenza A. In the final analysis, the solution of this problem may depend on identifying the exact conditions *at the site of infection* which make possible the initiation of disease by *Streptococcus pyogenes*.

SUMMARY

The findings indicate that : (*a*) freshly isolated cultures of *Streptococcus pyogenes* associated with the induction of an epidemic in man have mouse infectivity ; (*b*) the aerosol spray method described for the Swiss albino mouse is suitable for screening streptococci with potential epidemicity for man.

Throughout a 2-year period, the only streptococcal strains recovered within a Navy Recruit Training population showing mouse infectivity were type 19 organisms. These organisms gave rise to two streptococcal epidemics.

The epidemiological and laboratory observations together lend further support to the concept that the induction of streptococcal naso-pharyngeal infection is not purely a matter of the quantity of micro-organisms inhaled, but is determined, in part, at least, by qualitative differences in the bacteria contaminating the air.

The induction of streptococcal respiratory tract disease in the mouse appears to be dependent on an environmental as well as a bacterial factor. The possible nature of this environmental factor and some of its implications on the pathogenesis of human respiratory disease are discussed.

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PROPAGATION OF MOUSE ENCEPHALOMYOCARDITIS VIRUS IN ASCITES TUMOUR CELLS MAINTAINED *IN VITRO*

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MOUSE ascites tumours should provide an excellent source of cells for work with isolated animal virus/cell systems, since they consist of a large population ($1–2 \times 10^8$ cells per ml. of exudate) of separate cells floating free in the peritoneal cavity, and can easily be recovered and suspended in suitable media without the use of enzymes and other deleterious reagents. Although a number of recent papers have described the growth of viruses in such tumours *in vivo* (Koprowska and Koprowski, 1953; also see Moore, 1954, for review of literature), little use has been made of them so far for *in vitro* studies. For example, GD7 virus, NDV, and Bunyamwera virus have been shown to multiply in such cells *in vitro* by Sanders (1953), Moore and Diamond (1955) and Koprowski (1956), but in none of these cases was virus growth correlated with any observable cytological change.

In this paper we describe the growth, and maintenance by serial passage, of the virus of encephalomyocarditis (EMC virus) in ascites tumour cells *in vitro*. High yields of virus were obtained, and virus multiplication was accompanied by cytopathic destruction of the affected cells.

MATERIALS AND METHODS

Tumour

The ascitic form of the Krebs-2 mouse ascites carcinoma was used throughout. It was obtained from the Chester Beatty Research Institute in March 1953, and since that date has been maintained by serial passage in genetically heterogeneous Swiss mice supplied by an accredited dealer. Tumour passages were made at 10 to 12-day intervals by injecting 0·1 ml. of undiluted peritoneal exudate (containing $1–2 \times 10^7$ tumour cells) intraperitoneally into mice 6–8 weeks old.

Preparation of tissue cultures

Mice with well-developed ascites were killed by decapitation, and the ascitic fluid removed. Cells were first separated from the fluid by centrifuging for 10 min. at $210 \times g$, and the ascitic fluid decanted and saved. The cell layer, besides tumour cells, usually contained a variable proportion of red blood cells which could easily be removed by up to three low-speed washings in phosphate-buffered saline (P.B.S.; Dulbecco and Vogt, 1954). After the last washing the cells were suspended in culture medium, and adjusted to a concentration of about $1·0 \times 10^6$ cells/ml. The cell suspension was then dispensed in 1·5 ml. amounts in small, thin-walled, neutral glass culture bottles, 55 mm. long by 20 mm. in diameter. These had a volume of 12 ml. and were closed by a screw cap with a silicone rubber liner. The medium consisted of 65 per cent Earle's saline (Earle, Schilling, Stark, Straus, Brown and Shelton, 1943), 35 per cent mouse ascitic fluid, penicillin (Pen. G, crystalline sodium) 100 i.u./ml., and streptomycin (calcium chloride complex) 0·2 mg./ml. The ascitic fluid was stored at 4° for 3–4 days before use to allow clotting to take place, and the clot was then removed by centrifuging for 15 min. at $1880 \times g$. Cultures were infected directly after setting, using an inoculum of 0·15 ml., and were then incubated at 37° in a horizontal position. They were examined daily for cytopathic effect.

EMC virus

The virus was received originally from Dr. J. Warren, of the Department of Virus and Rickettsial Diseases, The Army Medical Center, Washington, as a lyophilized suspension of infected mouse brain in its 54th intracerebral mouse passage. Since then the virus has had 11 more intracerebral passages in mice at high dilution (10^{-5}, 10^{-6}). The stock virus with which culture passage was started consisted of a 10 per cent suspension of infected mouse brains in 10 per cent horse serum (N.H.S.) (in P.B.S.), stored at −70°. Infected cultures were harvested at the first sign of cytopathic change of the tumour cells, and frozen and thawed (−70°; 37°), and centrifuged before using the supernatant fluid for passage. In this way 10 passages were made in culture at high concentration, followed by 4 passages at limiting dilution. The tumour-propagated virus used in haemagglutination experiments was passed twice more in a culture system in which the cells were maintained in suspension (Sanders, unpublished).

Titration procedure

Titrations were performed in culture, and in mice, serial 10-fold dilutions in 10 per cent inactivated horse serum in P.B.S. always being used. Intracerebral titrations were performed, using 0·03 ml. volumes, in 3–4-week-old mice from the colony used to maintain the tumour. For intramuscular titrations only 21-day-old mice were used, 0·03 ml. volumes being injected into the right gastrocnemius muscle. Five mice (or cultures) were inoculated per virus dilution, and the 50 per cent end-points calculated by the method of Reed and Muench (1938). Mice and cultures were both scored daily, the former for the onset of specific symptoms, and the latter for the appearance of cytopathic change.

Haemagglutination technique

The method used was that of Gard and Heller (1951). All tests were carried out in M.R.C. pattern perspex haemagglutination trays.

RESULTS

Cytopathic changes

Under the conditions of culture described a sheet of tumour cells remained adherent to the wall of the culture vessel for about 6 days. The cells spread out on the wall of the vessel were large disc-shaped structures, with clear nuclei and cytoplasm, the latter showing a small number of prominent refringent granules (Fig. 1). After 6 days uninfected cultures began to undergo spontaneous changes, some cells detaching themselves from the glass, while others assumed bizarre forms; these included the development of spindle-, star-, and crescent-shaped cells, and the appearance of large intracellular vacuoles. The cells at this stage still possessed clear nuclei, while metabolism, indicated by a lowering of pH, still went on. That cell transformation to spindle forms is not a sign of degeneration is supported by Laznitski's finding (1952), that ascites cells of the S37 mouse sarcoma underwent similar changes *in vitro*, while at the same time showing a high proportion of mitotic figures. Acid production, especially in freshly-set cultures, was rapid, and whenever the pH reached about 6·8 as judged by the colour of the phenol red indicator, the medium was routinely replaced with fresh fluid.

Cells maintained in this way were able to support EMC virus multiplication, and at the same time underwent a characteristic cytopathic alteration. The first sign was a loss of their round outline; they then developed crinkled edges and began to shrink, the nuclei becoming pycnotic (Fig. 2). Finally, the cells shrivelled completely, became opaque, and detached themselves from the glass (Fig. 3). The pH of such degenerate cultures shifted rapidly to about 7·8. When

either mouse brain- or tumour-propagated virus was inoculated at high concentration, cytopathic alterations were evident after two days of incubation. For the highest dilutions the onset of the change was delayed up to 6 days. Although this is the time at which spontaneous changes begin to take place in uninoculated cultures (see above), the change in cell morphology in the latter is strikingly different from that produced by the virus, and could easily be distinguished from it.

Passage of virus in vitro

Passage of EMC virus *in vitro* was initiated by inoculating 0·15 ml. of a 10 per cent infected mouse brain suspension into 5 cultures. Two days later all cultures showed cytopathic alteration, and fluid from them was inoculated into a fresh series of cultures. In this way, 10 passages of the virus were made. The fluid harvested from the 10th passage was infective for mice, all 5 animals inoculated intracerebrally with undiluted culture material dying in less than 48 hr. The same material titrated *in vitro* contained $3{\cdot}4 \times 10^7$ T.C.D.$_{50}$/ml. A culture showing cytopathic change at the limiting dilution of 10^{-7} contained virus at a concentration of $6{\cdot}7 \times 10^6$ T.C.D.$_{50}$/ml. Two further passages of the latter material were made *in vitro* at limiting dilution before a pool was made from a large number of cultures. This pool contained $2{\cdot}1 \times 10^7$ T.C.D.$_{50}$ of virus/ml. When the same pool was titrated intracerebrally in mice it contained $1{\cdot}1 \times 10^8$ L.D.$_{50}$/ml., while for intramuscular inoculation the figure was $5{\cdot}3 \times 10^7$ L.D.$_{50}$/ml.

TABLE I.—*Titrations of Mouse Brain Virus and Krebs-2 Tumour Virus*

Virus	Titration system	Titre	I.D.$_{50}$/ml.*
Tumour virus	Intracerebral (i)	$10^{-6.5}$	$1{\cdot}1 \times 10^8$
	,, (ii)	$10^{-6.0}$	$3{\cdot}3 \times 10^7$
	Intramuscular (i)	$10^{-6.2}$	$5{\cdot}3 \times 10^7$
	,, (ii)	$10^{-6.6}$	$1{\cdot}3 \times 10^8$
	Tissue culture	$10^{-6.5}$	$2{\cdot}1 \times 10^7$
Mouse brain virus	Intracerebral (i)	$10^{-7.6}$	$1{\cdot}3 \times 10^8$
	,, (ii)	$10^{-8.2}$	$5{\cdot}3 \times 10^8$
	Intramuscular (i)	$10^{-8.8}$	$2{\cdot}1 \times 10^9$
	,, (ii)	$10^{-7.9}$	$2{\cdot}6 \times 10^8$
	Tissue culture	$10^{-7.5}$	$2{\cdot}1 \times 10^7$

* Expressed in terms of: undiluted culture fluid (tumour virus); 10 per cent infected brain (mouse brain virus).

Titration of mouse brain- and tumour-propagated virus

A series of titrations of mouse brain virus was made *in vivo* and *in vitro*. Using a single pool of virus grown in mouse brain tissue the following titrations were made: (*a*) Intracerebrally in mice; (*b*) intramuscularly in mice; and (*c*) in tumour cells in culture. A parallel series of titrations was made with tumour-propagated virus. The results of the titrations, summarized in Table I, show that tissue culture fluid contains about the same amount of virus as a 10 per cent suspension of infected mouse brain. Further, as a detector of virus the culture system possesses about the same degree of sensitivity as mice inoculated by the intracerebral route.

Haemagglutination and haemagglutination-inhibition experiments

Virus grown either in mouse brain or in Krebs-2 tumour cells in culture strongly agglutinated sheep erythrocytes under the conditions of the test. A 10 per cent suspension of a pool of infected mouse brains contained 190 haemagglutination (HA) units/ml. (geometric mean of 8 observations), while the pooled supernatant from a number of infected ascites tumour cell cultures had a virus content of 95 HA units/ml. (geometric mean of 4 observations).

After 16 passages in cultures of tumour cells, haemagglutination-inhibition tests were performed with tumour-propagated virus, using antisera prepared in rats against three other passage lines of EMC virus :— (*a*) Mouse brain virus ; (*b*) a strain grown in the Crocker mouse sarcoma 180 *in vitro* ; and (*c*) a virus of suspected human origin (" Fantz ")* which is serologically identical with mouse brain EMC. The results are summarized in Table II. The tests show no appreciable serological difference between any of the viruses tested.

TABLE II.—*Haemagglutination-inhibition of Mouse Brain and Krebs-2 EMC Virus*

Antiserum	Virus	H.-I. end-point
EMC (mouse brain virus) . .	Mouse brain .	1/200
	Krebs-2 .	1/100
Fantz (mouse brain virus) . .	Mouse brain .	1/1600
	Krebs-2 .	1/3200
EMC (Crocker virvs) . . .	Mouse brain .	1/1600
	Krebs-2 .	1/1600

DISCUSSION

These results show that EMC virus is capable of growing in a system of non-multiplying mammalian cells maintained *in vitro*. Ascites tumour cells removed from their hosts into a suitable medium do not show any significant number of mitoses, and it has been stated that under such conditions protein synthesis also ceases (Christensen, Riggs, Aspen and Mothon, 1956). Such cells, however, must retain sufficient synthetic ability to be able to support the growth of EMC virus.

Growth of EMC virus in Krebs-2 cells maintained *in vitro* does not appear to be restricted to a single cycle of virus multiplication, as has been suggested for Bunyamwera virus in these cells by Koprowski (1956). It rather resembles the propagation of the related Mengo virus in cells from the Ehrlich carcinoma, also described by this author. However, we did not observe the diminished titre of virus in cultures inoculated with high dilutions of virus, described by Koprowski for this system. For example, fluid from a culture inoculated with undiluted tissue culture virus had a virus content of $3{\cdot}4 \times 10^7$ T.C.D.$_{50}$/ml. ; the fluid from a culture inoculated with a 10^{-7} dilution of this same virus contained $6{\cdot}7 \times 10^6$ T.C.D.$_{50}$/ml., while two passages later fluid from a culture inoculated with a 10^{-5} dilution of infected culture fluid contained virus at a concentration of $2{\cdot}6 \times 10^7$ T.C.D.$_{50}$/ml.

EMC virus propagated in mouse brain has a high virulence for mice injected either intracerebrally or intraperitoneally. We did not find any alteration in infectivity for mice by either route following ten rapid passages of the

* This strain was obtained from the late Dr. G. W. Findlay who himself received it from Professor Hans Bielling who had isolated it in Germany.

virus in tumour cells at high concentration, followed by four passages at limiting dilution; nor was there any change in infectivity for the tumour cells during the same series of passages. Mouse brain virus was also unaltered in infectivity for mice following a comparable number of serial passages in various mouse organs *in vivo* and *in vitro* (Sanders and Taverne, unpublished). Koprowski (1956) has shown that Mengo virus can radically change its infectivity for mice following serial passage in cultures of Ehrlich ascites tumour cells. The first change recorded took place after 38 passages and it is possible that a similar change would have been detected if our passage series had been continued.

SUMMARY

EMC virus multiplies in cells of the Krebs-2 mouse ascites tumour maintained *in vitro*, and can be propagated serially in such a system.

Growth of the virus is associated with cytopathic destruction of the cells.

Tumour-propagated virus agglutinates sheep erythrocytes.

Haemagglutination-inhibition tests performed with EMC virus propagated in either mouse brain or tumour cells *in vitro*, show the two viruses to be serologically indistinguishable.

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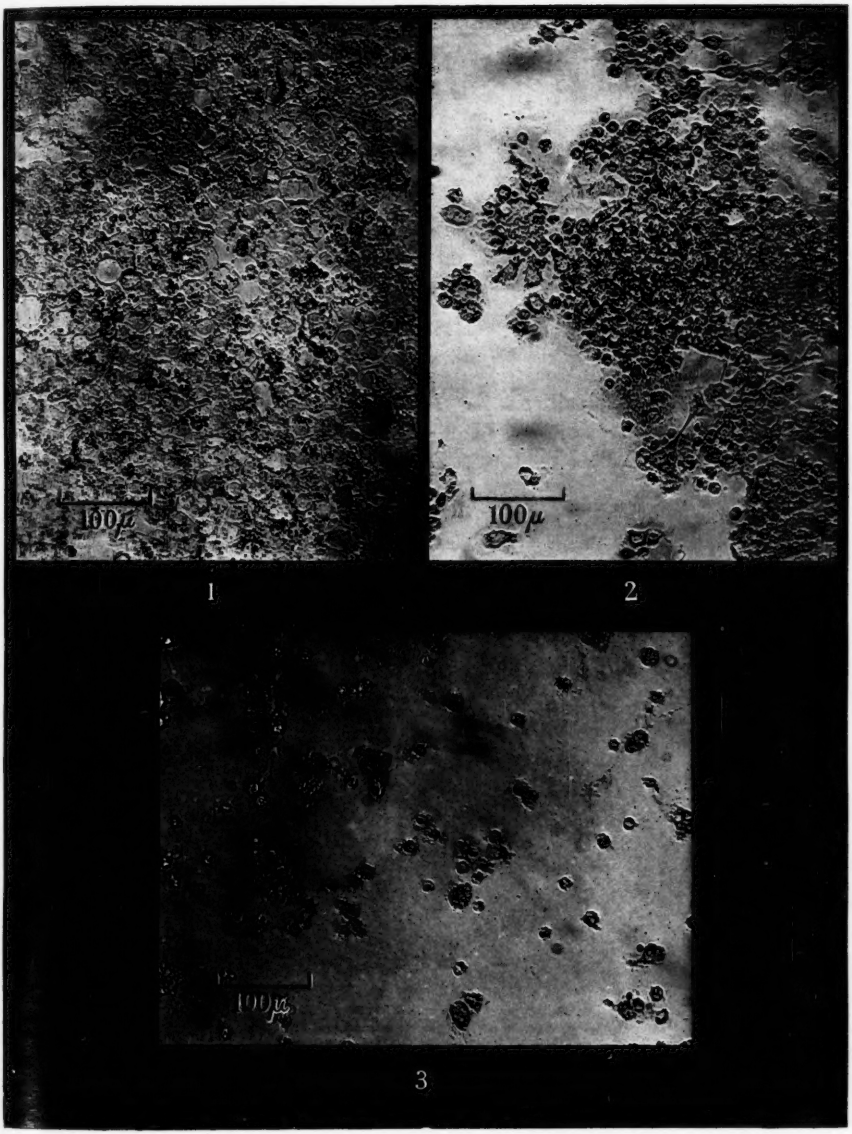
DESCRIPTION OF PLATE

Fig. 1.—Normal uninfected Krebs-2 ascites tumour cells. 48-hr. culture.

Fig. 2.—Krebs-2 ascites tumour cells infected 48 hr. previously with approximately 10^7 T.C.D.$_{50}$ of EMC virus.

Fig. 3.—Krebs-2 ascites tumour cells infected 72 hr. previously with approximately 10^7 T.C.D.$_{50}$ of EMC virus.



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A TECHNIQUE FOR THE STUDY OF SMALL EPIDERMAL WOUNDS

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DURING a comprehensive study of the physiology of mitosis (see reviews by Bullough, 1952, 1955), it was noted that cell division in the epidermis immediately adjacent to a wound is apparently influenced by factors other than those which govern the division of normal epidermal cells. Consequently an investigation of the physiology of the mitotic activity in such epidermis has been commenced. As a preliminary, it has been necessary to devise a technique for the infliction of a standard wound which may also be cultured *in vitro* according to the technique of Bullough and Johnson (1951). The present paper is concerned with this technique, and with the necessary preliminary study of the normal wound healing process.

METHODS

Adult male mice of the Kreyberg WLL strain, all between the ages of 3 and 6 months, were wounded by the infliction of 6 radial cuts made through the whole thickness of the ear and so placed that the larger blood vessels of the ear were undamaged. In this way 5 ear fragments approximately 4 mm. long and 3 mm. wide were semi-isolated, each fragment bearing a wound on both its edges. An ear fragment less than 3 mm. wide is liable to die and degenerate.

Ear wounds made in this way present several advantages, and in particular their edges do not contract. The ear fragments do not join together again and epidermal continuity is established by a meeting of the upper and lower epidermal sheets over the cut surface of the dermis. Thus the epidermal cells all have the same distance (the thickness of the ear, approximately 0·25 mm.) to migrate in order to re-establish continuity. The semi-isolated ear fragments are also ideal for eventual study by the *in vitro* technique.

At the required intervals the mice were killed by neck-pulling and the ear fragments were removed and fixed in either Zenker-formol or Dubosq-Brasil. The ear fragments were orientated so that transverse sections of both wounds were obtained. The sections, 7 μ thick, were stained in Mayer's haemalum and Gurr's B.A. eosin for the study of mitosis, in Masson trichrome stain for observations on wound closure, and by the Bauer-Feulgen or the periodic acid-Schiff method of McManus (1948) for the study of glycogen.

In all cases only one ear was wounded, the other being used for a comparative study of normal epidermis.

RESULTS

Wound Closure

The details of the closure of epidermal wounds have been described adequately elsewhere (Arey, 1936; Firket, 1951; Needham, 1952) so that it is necessary to mention here only the major events and their time relations.

Wound after 12 hr.—During the first 12 hr. necrosis of the epidermis and underlying tissues occurred to a depth of about 0·2 mm. Leucocytes, appearing in abundance in the now swollen capillaries, began to invade the dermal tissue and to migrate towards the wounded edge.

Wound after 24 *hr.*—After 24 hr. a heavy accumulation of leucocytes had developed beneath the necrotic regions, and a further invasion of leucocytes apparently continued until the wound finally closed. Also after about 24 hr. the epidermal cells adjacent to the wound began to swell and to lose their affinity for stains. The region particularly affected in this way had a width of only about 0·3 mm.

Wound after 36 *hr.*—Between 24 and 36 hr. the epidermal cells immediately adjacent to the wound began to migrate across the exposed surface of the dermis to meet those coming from the opposite ear surface. This migration took place beneath the now heavy accumulation of leucocytes which lay beneath the scab. The distance covered by the migrating cells was fairly constant, but since the ear is slightly thinner towards its outer edge, wound closure was always achieved slightly earlier in this distal region.

Wound after 48 *hr.*—By 48 hr. the two sheets of migrating epidermal cells had met, and the wounds were closed along their full lengths. Also by 48 hr. the epidermal cells in the region which formed the original edges of the wound began to undergo mitosis. It was found during normal healing that the beginning of active mitosis coincided with the closure of the wound, but if, as occasionally happened, wound closure was delayed, active mitosis still began after about 48 hr. Thus the closure of the wound by cell migration is not a prerequisite for the development of active mitosis. It should be noted that mitoses were never observed in the migrating sheets of epidermal cells, but after these sheets had met and fused cell division did take place in the newly-formed epithelium.

Older wounds.—During the third day the scab began to loosen and fall off. By this time the underlying epidermal layer was thick, and after 6 days it had formed a fully differentiated epidermis.

Epidermal Mitotic Activity

Because of the existence of a diurnal mitotic rhythm in normal epidermis, precautions were taken lest a similar rhythm should also exist in the mitotic activity of epidermis adjacent to a wound. The time at which the ear wounds were inflicted was arranged so that, after the appropriate period had elapsed, the mice could be killed during their normal afternoon sleep. This precaution was later proved to be unnecessary since in fact no diurnal mitotic rhythm does exist in wounded epidermis.

To facilitate counting, each mouse was injected subcutaneously with 0·1 mg. colchicine 5 hr. before death. By this means all those mitoses developing during the final 5 hr. were arrested in the metaphase. It was found that the marked increase in the mitotic activity was confined within the first millimetre length of epidermis adjacent to the cut, and therefore the mitotic counts were also confined to this region.

For each wound 10 such counts were made and an average taken. From the many figures so obtained from each experimental group, a grand average and standard error were derived. From each sample of normal epidermis, 5 counts were made of the numbers of mitoses present in unit section lengths of 1 cm. The average then taken was divided by 10 to adjust the figure to represent a section length of 1 mm. From the many figures so obtained for each group, a grand average and standard error were derived.

TABLE I.—*Effect of Age of Wound on Mitotic Activity*

Number of animals used	Age of wound	Number of wounds studied	Average number of mitoses
5	12 hr.	59	1·05±0·09
5	24 ,,	39	1·08±0·10
5	36 ,,	49	1·8 ±0·15
4	2 days	35	8·67±0·37
5	3 ,,	36	7·25±0·38
4	4 ,,	28	5·55±0·36
5	5 ,,	44	2·5 ±0·25
5	6 ,,	35	2·0 ±0·15

Average numbers of mitoses arrested by colchicine in 5 hr. in ear epidermis immediately adjacent to a wound. Counts were made on unit lengths of 1 mm. of epidermis cut 7 μ thick.

The mitotic activity in 12, 24, and 36 hr. wounds, as well as in 2, 3, 4, 5, and 6 day wounds was studied, and the results are shown in Table I. It is clear that the mitotic rate rises suddenly on the second day, remains high during the third day, and falls steadily thereafter. There is no significant difference between the high mitotic rates seen on the second and third days.

TABLE II.—*Effects of Rest and Exercise on Mitotic Activity*

Number of animals	Number of wounds or observations	State of animal	Time of experiment (hrs.)	Average number of mitoses	Age of wound (days)
Wounded epidermis—					
4	29	Period of activity and feeding in normal surroundings	15.30–20.30	11·3±0·50	2
5	28	Kept exercising in revolving box	12.00–17.00	11·3±0·66	3
4	35	Period of sleep in normal surroundings	12.00–17.00	8·67±0·37	2
5	36	Period of sleep in normal surroundings	12.00–17.00	7·25±0·38	3
Normal epidermis—					
2	12	Period of activity and feeding in normal surroundings	15.30–20.30	0·07±0·013	—
5	23	Kept exercising in revolving box	12.00–17.00	0·08±0·015	—
2	12	Period of sleep in normal surroundings	12.00–17.00	0·76±0·10	—

Average numbers of mitoses arrested by colchicine in 5 hr. in ear epidermis immediately adjacent to a wound and in normal ear epidermis. In epidermis adjacent to a wound counts were made on unit lengths of 1 mm., while in normal epidermis counts were made on unit lengths of 1 cm. and the results were divided by 10. All sections were cut at 7 μ.

Experiments were next undertaken to determine whether a diurnal mitotic rhythm exists in this wound-stimulated epidermis (Table II). It was confirmed that a marked rhythm does exist in normal epidermis with the highest number of mitoses present during rest or sleep and the lowest during muscular exercise in a revolving box. No such variations were found in the mitotic rate of wounded epidermis. Even when the mice were kept in a slowly revolving box for 6 hr., the rate of mitotic activity in the epidermis adjacent to the wound remained as high as in the resting controls.

TABLE III.—*Effect of Starvation on Mitotic Activity*

Number of animals	Number of wounds or observations	State of animal	Average number of mitoses
		Wounded epidermis	
6	35	Control	8·67±0·37
6	32	Starved 24 hr.	1·09±0·97
		Normal epidermis	
3	12	Control	0·76±0·10
3	12	Starved 24 hr.	0·04±0·04

Average numbers of mitoses arrested by colchicine in 5 hr. in ear epidermis immediately adjacent to a wound and in normal ear epidermis. In epidermis adjacent to a wound counts were made on unit lengths of 1 mm., while in normal epidermis counts were made on unit lengths of 1 cm. and the results were divided by 10. All sections were cut at 7 μ.

To determine the effects of diet, mice were starved for 24 hr., and the results are shown in Table III. Confirmation was obtained that such treatment results in a deep depression of mitotic activity in normal epidermis (Bullough and Eisa, 1950). A similar depression was also seen in the mitotic activity of cells adjacent to a wound.

Glycogen Deposition

It is well known that glycogen is frequently deposited in the neighbourhood of healing wounds, and in view of the known relationship between normal epidermal mitotic activity and the supply of energy in the form of glucose from the blood (Bullough, 1952), it was important to determine the position of glycogen deposits in the wounded ear. It was found that the first signs of glycogen appeared in the 24 hr. wound, the granules lying within the accumulating leucocytes at the base of the necrotic zone. At no time was glycogen demonstrated in the non-migrating epidermal cells, but in the migrating cells isolated granules were very rarely seen. By 36 hr. the glycogen deposition in the leucocytes was heavy, but it disappeared progressively as epidermal cells migrated beneath the leucocyte zone. In the fully closed wound of 48 hr. and older no glycogen could be demonstrated.

CONCLUSIONS

This analysis has been made as a preliminary to a study of the physiology of mitosis in epidermis adjacent to a wound. It is concluded that fragments of ear detached on the third day, when wound closure is complete and when mitotic activity is high, offer the best material for an *in vitro* study by the technique of Bullough and Johnson (1951). Such ear fragments contain no glycogen deposits which could be used as a source of energy in culture experiments.

SUMMARY

A description is given of the day-by-day variations in the mitotic activity of epidermal cells adjacent to a wound inflicted in the ear of an adult male mouse. Extremely high mitotic activity was found during the second and third days

after wounding. This high mitotic activity was confined to a 1 mm. length of epidermis immediately adjacent to the damaged region.

The greatly increased mitotic activity which developed after epidermal wounding was not subject to a diurnal rhythm and was not inhibited by heavy muscular exercise. Diet may however be important since mitotic activity was found to be depressed during starvation.

Glycogen was present in the leucocyte zone before wound closure was complete. After wound closure no glycogen was visible.

Our thanks are due to the British Empire Cancer Campaign and to Organon Laboratories Ltd. for the grants which made this research possible.

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THE ENERGY RELATIONS OF EPIDERMAL MITOTIC ACTIVITY ADJACENT TO SMALL WOUNDS

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IT has been shown that the supply of energy in the form of glucose is a critical factor limiting the mitotic activity of normal epidermis (Bullough, 1952). Since the mitotic activity of epidermal cells adjacent to a wound is far higher than that of normal cells (Bullough, 1946), the question arises whether this increased rate is due solely to a greater glucose intake from the enlarged capillary network, or whether some other factor altogether is responsible. The present paper is designed to throw light on the changed metabolism of epidermal cells adjacent to a small wound and in particular on the energy relations of the mitotic activity.

MATERIALS AND METHODS

The mice used and the wounding technique employed have been described in the previous paper (Bullough and Laurence, 1957), while details of the *in vitro* techniques have been given by Bullough and Johnson (1951).

Except in the first experiment, ears wounded 3 days previously were used. Such wounds were already closed by a migration of epidermal cells, there were no glycogen deposits, and the mitotic rate was high. Only one ear was wounded, the other being used for a comparative study of the reactions of normal epidermis.

The fragments of ear, normal and wounded, were incubated in Warburg flasks in phosphate-buffered saline for 1 hr. at 38° to eliminate those mitoses already present in the epidermis when it was separated from the body. To arrest the newly-developing mitoses in the metaphase, 0·16 mg. colchicine in 0·04 ml. saline was added to the 4 ml. saline present in each flask, and incubation was continued for a further 4 hr., except in the first experiment when, for special reasons, incubation with colchicine was continued for a further 5 hr.

The methods of assessing the mitotic activity have been described in the previous paper (Bullough and Laurence, 1957).

RESULTS

Effects of age of wound on mitotic activity in vitro

During the healing of a wound the adjacent epidermal cells enjoy a greatly increased blood supply, and it is possible that this may itself provide the full explanation for the greatly increased mitotic rate. To test this thesis a comparison was made between the mitotic activity *in vivo* and that developing in the absence of a blood supply *in vitro*. In the *in vivo* experiments each mouse was injected subcutaneously with 0·1 mg. colchicine and killed 5 hr. later. In the *in vitro* experiments the wounded ear fragments were cultured for 1 hr. before the colchicine was added, and the experiment was continued for a further 5 hr. Wounds aged between 1 and 6 days were used, and the results are shown in Table I. Results for normal ear epidermis taken from the same animals are also included.

TABLE I.—*Effect of Age of Wound on Mitotic Activity*

In vivo			*In vitro*		
Age of wound in days	Number of wounds studied	Average number of mitoses	Age of wound in days	Number of wounds studied	Average number of mitoses
1	59	1·08±0·09	1	22	1·59±0·17
2	35	8·67±0·37	2	12	14·40±0·74
3	36	7·25±0·38	3	22	10·87±0·57
4	28	5·55±0·36	4	26	6·51±0·76
5	44	2·50±0·25	5	20	1·59±0·27
6	35	2·00±0·15	6	14	0·80±0·27
Average number of mitoses in normal epidermis = 0·76±0·10			Average number of mitoses in normal epidermis = 1·36±0·06		

Average numbers of mitoses arrested by colchicine in 5 hr. in ear epidermis immediately adjacent to a wound and in normal ear epidermis. Counts refer to unit lengths of 1 mm. epidermis cut 7 μ thick. *In vitro* the epidermis was incubated at 38° in a phosphate-buffered saline with 0·02 M glucose and an oxygen gas phase.

It is obvious from this Table that the mitotic rate developing *in vitro* is similar to that developing *in vivo*. Activity was low after only 24 hr., but rose rapidly to a maximum on the second and third days. Thereafter it steadily decreased. It is interesting that in both normal epidermis and in epidermis adjacent to a wound the mitotic rate developing *in vitro* considerably exceeded that seen *in vivo*. The greater activity of the cells maintained *in vitro* may have been due to the optimum concentration of glucose and to the oxygen gas phase.

However, the most important observation is that *in vitro* as well as *in vivo* the wounded epidermis developed a far higher mitotic rate than did the normal epidermis. This effectively disposes of any hypothesis that the greater activity of the epidermal cells adjacent to a wound is due solely to their richer blood supply. Evidently in the neighbourhood of a wound the cells undergo some change in their metabolic pattern resulting in the development of a higher mitotic potential.

A more detailed analysis of this increased mitotic activity was next made. The 1 mm. lengths of epidermis previously studied were divided into three equal parts and the mitoses present in each were counted separately. The results are summarized in Table II. It will be seen that in wounds of all ages the highest numbers of mitoses were in that region immediately adjacent to the scab. Evidently the influence of the wound on the metabolic pattern of the adjacent cells falls steeply with increasing distance and thus gives the impression of a chemical diffusion effect.

TABLE II.—*Variation in Mitotic Activity with Distance from Wound*

Number of animals used	Age of wound in days	Number of wounds studied	Average number of mitoses		
			1	2	3
5	1	50	0·46±0·09	0·32±0·078	0·34±0·094
4	2	40	4·22±0·37	3·15±0·36	1·57±0·26
5	3	50	3·9 ±0·27	1·76±0·27	1·40±0·22
4	4	40	2·72±0·38	1·42±0·25	0·80±0·26
5	5	48	2·10±0·29	0·71±0·17	0·27±0·05

Average numbers of mitoses arrested by colchicine in 5 hr. in ear epidermis immediately adjacent to a wound. The unit length of 1 mm. of epidermis cut 7 μ thick, referred to in Table I, has been divided into 3 equal parts, unmber 1 being adjacent to the wound and number 3 most distant from it.

Respiration and mitosis in vitro

Since the respiratory rate of the epidermis has a direct and powerful effect on the mitotic rate (Bullough, 1952), the question arises whether the suggested change in the metabolic pattern of the cells adjacent to the wound may involve fundamental changes in carbohydrate metabolism. Normal epidermal cells cannot undergo mitosis unless they are actively respiring (Bullough, 1952), although evidently embryonic fibroblasts are able to divide with the support of glycolysis alone (Pomerat and Willmer, 1939). The relation of the mitotic activity of epidermal wounds to glycolysis and respiration was therefore considered next.

A series of experiments was performed to test the value of glucose and fructose as substrates and of oxygen in the gas phase. In normal epidermis glucose is a good substrate, but, since the glucokinase reaction may become limiting, fructose, which by-passes this reaction, is better (Bullough, 1952). The results of these experiments are shown in Tables III and IV. These show that, with a gas phase of oxygen, the presence of glucose has the same stimulating effect on the mitotic rate in the vicinity of a wound as it has in normal epidermis. In both cases, too, the presence of fructose may exert an even greater stimulating effect, although in the normal epidermis the difference recorded here is not statistically significant.

TABLE III.—*Effect of Carbohydrate Substrate on Mitotic Activity*

	Epidermis adjacent to wound		Normal epidermis	
Substrate	Number of wounds studied	Average number of mitoses	Number of ear fragments	Average number of mitoses
Saline alone	49	1·70±0·35	29	0·33±0·008
Saline + 0·02 M glucose	50	7·00±0·60	49	1·10±0·054
Saline + 0·02 M fructose	35	9·50±1·12	19	1·21±0·078

Average numbers of mitoses arrested by colchicine in 4 hr. in fragments of ear epidermis immediately adjacent to a wound and in normal ear epidermis. Ear fragments were incubated at 38° in a phosphate-buffered saline with an oxygen gas phase. Counts refer to unit lengths of 1 mm. of epidermis cut 7 μ thick.

TABLE IV.—*Effect of Gas Phase on Mitotic Activity*

	Epidermis adjacent to wound		Normal epidermis	
Gas phase	Number of wounds studied	Average number of mitoses	Number of ear fragments	Average number of mitoses
Nitrogen	34	0·16±0·05	15	0·04±0·008
Air	53	0·53±0·10	20	0·45±0·059
Oxygen	50	7·00±0·60	49	1·10±0·054

Average numbers of mitoses arrested by colchicine in 4 hr. in fragments of ear epidermis immediately adjacent to a wound and in normal ear epidermis. Ear fragments incubated at 38° in a phosphate-buffered saline with 0·02 M glucose. Counts refer to unit lengths of 1 mm. of epidermis cut 7 μ thick.

Similarly, in the presence of glucose as substrate, there is almost no activity in a gas phase of nitrogen, moderate activity in air, and maximum activity in oxygen. The results in both Tables emphasize that, although the mitotic activity

of epidermis adjacent to a wound is always higher than that in normal epidermis, this higher activity is equally dependent on active respiration. There is no evidence that any significant activity can continue in anaerobic conditions.

To test this conclusion still further a series of experiments was carried out involving the use of a number of inhibitors of carbohydrate metabolism and of energy transfer. The results obtained with cyanide and azide, inhibitors of the cytochrome system, with malonate, a competitive inhibitor of the oxidation of succinate to fumarate, and with fluoride, an inhibitor of glycolysis, are shown in Table V, and with dinitrophenol, which inhibits aerobic phosphorylation (Loomis and Lipmann, 1948), in Table VI.

The results given in these Tables show that all these various inhibitors of carbohydrate metabolism and of energy transfer act as powerful inhibitors of

TABLE V.—*Effects of Carbohydrate Inhibitors on Mitotic Activity*

		Epidermis adjacent to wound		Normal epidermis	
Inhibitor	Concentration	Number of wounds studied	Average number of mitoses	Number of ear fragments	Average number of mitoses
Sodium cyanide	0	6	12·73±2·76	4	1·420±·229
	0·0005 M	9	0·11±0·035	4	0·0150±·030
	0·001 M	13	0·07±0·015	5	0·0080±·005
	0·005 M	5	0·20±0·017	5	0
Sodium azide	0	7	7·90±0·94	5	1·78±0·19
	0·0001 M	7	8·31±1·09	5	0·49±0·057
	0·0005 M	6	0·67±0·051	5	0·06±0·028
	0·001 M	8	cells necrotic	5	cells necrotic
Sodium malonate	0	7	8·20±1·43	4	0·96±0·120
	0·01 M	7	1·23±0·40	4	0·22±0·072
	0·02 M	6	0·05±0·05	5	0·18±0·012
	0·03 M	8	0·05±0·04	4	0·01±0·013
Sodium fluoride	0	5	9·06±1·51	5	1·01±0·098
	0·0005 M	6	1·28±0·48	5	0·02±0·002
	0·001 M	6	0·25±0·13	5	cells necrotic
	0·005 M	7	0	5	" "

Average numbers of mitoses arrested by colchicine in 4 hr. in fragments of ear epidermis immediately adjacent to a wound and in normal ear epidermis. Ear fragments incubated at 38° in a phosphate-buffered saline with 0·02 M glucose and an oxygen gas phase. Counts refer to unit lengths of 1 mm. of epidermis cut 7 μ thick.

TABLE VI.—*Effect of 2 : 4 Dinitrophenol on Mitotic Activity*

	Epidermis adjacent to wound		Normal epidermis	
Concentration of 2 : 4 dinitrophenol	Number of wounds studied	Average number of mitoses	Number of ear fragments	Average number of mitoses
0	9	6·17±1·52	5	1·12±0·041
0·00001 M	6	6·50±1·64	5	0·52±0·077
0·00005 M	6	6·30±0·75	4	0·1±0·036
0·0001 M	6	0·47±0·22	5	0
0·0005 M	9	0	5	0

Conditions as specified in Table V.

epidermal mitotic activity, whether normal or adjacent to a wound. In most cases too the effective concentration of the inhibitors is similar in both types of epidermis.

DISCUSSION

From these results it is evident that the high mitotic activity adjacent to an epidermal wound is not due basically to the increased richness of the blood supply, although it may be doubted whether such high activity could develop *in vivo* in the absence of a rich capillary network. Even with the enhanced blood supply the activity developing *in vivo* is not nearly so great as that which can be induced by optimum conditions *in vitro*.

Although it appears that some sudden change in the metabolic pattern develops in the uninjured cells adjacent to a wound, it is clear that this change involves no major alteration in the method of energy production within the cells. In preparing for division both normal cells and cells adjacent to a wound are dependent on the energy derived from the Krebs cycle. Sufficient supplies of glucose and oxygen are essential.

It is most probable that, in order to support their far greater activity, the cells close to a wound must absorb far larger quantities of glucose and oxygen than do normal epidermal cells. In normal cells there is evidence that the glucokinase enzyme system, which may be situated in the cell wall, does not operate with full efficiency (Bullough, 1952), and it may be suggested that the greatly increased activity of cells adjacent to wounds may be in some degree dependent on a more active cell surface glucokinase system. However, it does not appear that this can be more than a partial explanation. When normal epidermis is providedwith an easily assimilable energy source such as lactate, so by-passing the relatively inefficient glucokinase system, there may be some increase in the mitotic rate but the degree of this increase does not approach that seen in the vicinity of a wound.

Thus it appears that the high mitotic activity of the epidermal cells adjacent to a wound must be due to some more fundamental metabolic change of which the evidently enhanced rate of glucose uptake is a side-effect. It should be noted that the observed metabolic change does not extend far from the edge of the wound and that, even within this short distance, there is a sharply falling mitotic gradient, the highest activity being immediately adjacent to the wound. This mitotic gradient gives the impression of being based on the diffusion gradient of some substance emanating from the region of the wound.

It may be suggested that, in optimum conditions, cells will continue to show the most active mitosis until they are restrained from doing so. Those cellular changes which are associated with differentiation involve the imposition of such a restraint. We are still far from an understanding of these restraining forces although some indications of their possible nature have emerged from the work of Weiss (1952). This suggests that the tissues of an organ, as for instance the liver, secrete substances into the body which, on reaching an optimum concentration, act back to inhibit the further growth of those tissues. With the partial excision of the tissues, as in partial hepatectomy, the resulting fall in concentration of the tissue inhibitors within the body space allows growth to recommence to give rise to what is known as compensatory hypertrophy. On this hypothesis it could be argued that a similar local lack of inhibitors, or perhaps more probably a local neutralization of inhibitors, occurs in the vicinity of a wound to permit increased mitotic activity to develop.

SUMMARY

When wounded epidermis is separated from the body and incubated for 5 hr. *in vitro*, the same high rate of mitotic activity develops two or three days post-wounding as is the case *in vivo*.

Both in wounded and in normal epidermis the mitotic rate developing *in vitro* is considerably higher than that seen *in vivo*.

The highest mitotic rate is always found in those epidermal cells immediately adjacent to the wound and the activity falls steeply with increasing distance.

The high mitotic activity of the cells adjacent to a wound is dependent on adequate supplies of oxygen and of a suitable carbohydrate substrate such as glucose or fructose. Known inhibitors of carbohydrate metabolism and of energy transfer are also inhibitors of the mitotic activity.

It is concluded that, although epidermal cells adjacent to a wound develop a mitotic rate which is about ten times that of normal epidermis, this type of cell division is essentially similar to that of normal cell division in its dependence on energy production by respiration.

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THE TOXICITY OF SOME BACTERIAL FILTRATES FOR MICE PRE-INFECTED WITH *EPERYTHROZÖON COCCOIDES*

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CONCOMITANT or previous infection of mice with *Eperythrozöon coccoides*, a harmless blood parasite of mice, greatly enhances the pathogenic action of mouse hepatitis virus (MHV1) (Niven, Dick, Gledhill and Andrewes, 1952); this fact has provided a useful method of studying the action of the virus (Gledhill and Niven, 1955). We now report another peculiarity of mice recently infected with *E. coccoides*. During the passage of murine liver suspensions by intraperitoneal inoculation, undertaken as a routine measure to ensure that a newly introduced group of mice were not carriers of ectromelia virus, hepatitis appeared which was due to a virulent strain of *Salmonella typhi-murium*. It was observed that the organism killed mice infected with *E. coccoides* much more quickly than it killed normal mice. Since *E. coccoides* enhances the pathogenic action of mouse hepatitis virus (MHV1) by its effect on the liver, it seemed possible that this parasite might influence the pathogenicity of the strain of *Salm. typhi-murium* (termed S_4) by increasing susceptibility to the toxic effect of this organism rather than by increasing its rate of multiplication. Experiments described in this paper show that formalin-inactivated 20-hour cultures are lethal to mice pre-treated with *E. coccoides* and that the toxic principle is present in cultures as a filtrable substance. Furthermore, filtrates of cultures of other related Gram-negative bacteria are toxic for mice infected with *E. coccoides* while filtrates of cultures of the Gram-positive bacteria tested were not. Filtrates of cultures of the Gram-negative bacteria tested are practically non-toxic for normal mice.

METHODS

Unless otherwise stated, we used throughout weanling mice aged 20–23 days of the VS strain which is not usually infected with *E. coccoides*. The bacteria were grown in Wright's broth (pH 7·0) for 18–20 hr. at 37°. Formalin was then added to the culture to give a final concentration of 0·25 per cent and the mixture was incubated at 37° for a further 24 hr. to inactivate the bacteria. As a preliminary to filtration, the cells were deposited by centrifugation for 10 min. in an angle centrifuge at about 3000 r.p.m. For most purposes the supernatant was filtered through a gradocol membrane of 1·5 μ average pore diameter (A.P.D.) standing on a membrane of 0·54 μ A.P.D. Filtrates were usually injected into mice intraperitoneally and the condition of the mice observed after 6, 12, 24, 48 and 72 hr., when the experiments were terminated. Results were judged chiefly by the total mortality within 72 hr. and to a lesser extent by illness during the first 24 hr.

Dr. Joan Taylor has confirmed that S_4 is a strain of *Salm. typhi-murium* (antigenic structure 0 = 4, 12; H = i – 1, 2). High dilutions of culture are lethal for mice and its virulence equals that of virulent strains. Other species and strains of bacteria were used as hereunder:

Salm. typhi-murium, 3 strains; one virulent, D_2 from Dr. J. S. Paterson; two avirulent, Toronto and M206 from Dr. D. Hobson.

Salmonella limete from Dr. J. S. Paterson.

Salmonella cholerae-suis, Salmonella paratyphi, Salmonella enteritidis, Salmonella newport and *Salmonella london* from Dr. Joan Taylor.

Bacterium coli 4 strains; one of uncertain origin; D433, D2101 and E793 from Dr. Joan Taylor.

Strains of *Shigella—Sh. shigae, Sh. flexneri* and *Sh. boydii* (2 strains) from Dr. K. Patricia Carpenter.

Staphylococcus aureus, coagulase-positive strain from Dr. J. H. Humphrey.

Streptococcus pyogenes, group A strain from Dr. H. Rogers.

Erysipelothrix rhusiopathiae, mouse virulent strain Ru.

Mice were infected with *E. coccoides* by injecting them with 10^{-7} dilution of spleen suspension taken from mice which had been themselves inoculated a week before with *E. coccoides* spleen suspension diluted 10^{-8}; the inoculation of bacteria or bacterial filtrate was given 120–128 hr. after that of *E. coccoides*. This interval between the inoculation of parasite and bacteria or filtrate was selected because it was considered likely to be the most effective by analogy with the results of experiments with mouse hepatitis virus (Gledhill, 1956) and this was confirmed in experiments to be described. The mice thus pre-treated with *E. coccoides* will be termed " pre-treated mice " and mice not so treated will be termed " normal mice ".

RESULTS

The toxicity of filtrates of S_4

In order to test whether an inactivated growth of S_4 was toxic for pre-treated mice, an 18–20 hr. growth was treated with formalin to give a concentration of 0·25 per cent and after further incubation at 37° for 24 hr. it proved to be sterile. Some of this bacterial suspension in doses of 0·3 ml. was injected intraperitoneally into normal and pre-treated mice. The rest of the suspension was centrifuged to deposit the cells, and the supernatant was injected in the same dose into normal and pre-treated mice. One fraction of the deposited cells was re-suspended in a volume of broth equal to that in which they were dispersed before centrifugation and another was similarly re-suspended in supernatant. Both these bacterial suspensions were injected into normal and pre-treated mice. The results of this experiment are presented in Table I from which it is clear that the supernatant of

TABLE I.—*Enhanced Toxicity of Supernatant from Formalin-inactivated Growth of* Salm. typhi-murium (*Strain* S_4) *for* E. coccoides *Pre-treated Mice*

Material*	Mice pre-treated with *E. coccoides*		Mice not pre-treated with *E. coccoides*	
	Condition 12 hr. after inoculation	Mortality 72 hr. after inoculation	Condition 12 hr. after inoculation	Mortality 72 hr. after inoculation
Formolized whole culture	9† 1VS**	10/10	3SS 7N	0/10
,, supernatant	6† 4S	10/10	1S 2SS 7N	0/10
,, cells + broth	1† 1VS 2SS 6N	3/10	10N	0/10
Recombined cells+supernatant	6† 4S	10/10	3SS 7N	0/10
Formolized broth	5N	0/5	5N	0/5

* Mice were inoculated (0·3 ml. intraperitoneally) with materials from 20 hr. growth *Salm. typhi-murium* strain S_4 inactivated by incubation with formalin in a final concentration of 0·25 per cent.

** Symbols for condition of mice: † = dead; VS = very ill; S = ill; SS = slightly ill; N = normal.

the inactivated growth contains a substance lethal for pre-treated mice and practically non-toxic for normal mice. The cells had some toxicity for pre-treated mice which was, however, much less than that of the supernatant. Another

TABLE II.—*Enhanced Toxicity of Filtrate and Supernatant of Growths of* Salm. typhi-murium (*Strain S_4*) *for* E. coccoides *Pre-treated Mice*

Material (from 20 hr. growth of *Salm. typhi-murium*, strain S_4)	Dose of inoculum (ml.)	Mice pre-treated with *E. coccoides*		Mice not pre-treated with *E. coccoides*	
		Condition 11 hr. after inoculation	Mortality 72 hr. after inoculation	Condition 11 hr. after inoculation	Mortality 72 hr. after inoculation
Filtrate (540 mμ A.P.D.) of unformolized growth	0·3	5† 1VS 1SS 1N**	6/8	1SS 7N	0/8
Supernatant of formolized growth*	0·3	5† 5S	5/8	8N	0/8
Supernatant of formolized growth*	0·03	2VS 2SS 4N	2/8	N.T.	N.T.
Supernatant of formolized growth*	0·1	1† 3VS 3S 1N	5/8	8N	0/8
Supernatant of formolized growth*	0·3	3† 4S 2SS	6/9	1SS 8N	0/9
Supernatant of formolized growth*	0·6	N.T.	N.T.	6SS 2N	0/8
Supernatant of formolized growth heated at 70° for 30 min.	0·3	2† 4S 2SS	5/8	1SS 7N	0/8

* Formolized growth inactivated by incubation with 0·25 per cent formalin for 24 hr.
** Symbols for condition of mice: † = dead; VS = very ill; S = ill; SS = slightly ill; N = normal. N.T. = not tested.

experiment was carried out to show that the toxic principle was filtrable through a gradocol membrane impermeable to the bacteria and that filtrates of living growth contained the principle present in formolized growth. At the same time, varying doses of formolized supernatant heated to 70° for ½ hr. were introduced into treated and normal mice. The results are shown in Table II. It will be seen that none of the inocula used produced significant symptoms in normal mice whereas 60–80 per cent of pre-treated mice of all groups (except that given the smallest dose of toxic agent, *viz.*, 0·03 ml.) were killed. Further, it appeared that the toxic principle was not heat-labile. The results of the experiment are less clear-cut than those presented in Table I in which all pre-treated mice were killed by toxin. Since the failure to kill all pre-treated mice in the experiment of Table II could not be correlated with the dose of toxin given, it was suspected that, at the time when the toxin was injected, the concentration of *E. coccoides* in the pre-treated mice of this experiment was not optimal for the production of the enhancing action. This possibility was tested in the following experiments.

The effect of filtrate injected at varying times after inoculation with E. coccoides

Spleen suspension at a dilution of 10^{-7} was injected into 9 groups of 10 mice each. Exactly 24 hr. afterwards one group was inoculated with filtrate, a second group at 48 hr. and so on at daily intervals until all the groups had been inoculated. Blood smears were taken from each mouse of every group at the time of its inoculation with toxin and, by suitable marking of the mice of each group, observations on the *E. coccoides* present in each blood smear could be related to the reaction towards filtrate of the mouse from which it had been taken. A summary of the results is shown in Table III. It will be seen that the filtrate did not affect the first three groups of mice. Mice of group 4 were the first in which *E. coccoides* was plentiful in smears and although these mice became ill following the injection of filtrate,

TABLE III.—*The Relationship of Mouse Response to Culture Filtrate to the Time of Inoculation and Abundance of* E. coccoides *in Blood Smears*

Time between *E. coccoides* inoculation and filtrate inoculation in days	Abundance of *E. coccoides* in blood smears at the time of inoculation with filtrate	Result of inoculation with filtrate	
		Number ill	Number dead
1	Absent from all smears	0/10	0/10
2	" " "	0/10	0/10
3	Absent from 4 smears, present (very scanty) in 6	0/10	0/10
4	Present in all smears, abundant in 8	10/10	0/10
5	Present in all smears, abundant in 9	10/10	5/10
6	Present in all smears, abundant in 9	10/10	6/10
7	Present in all smears, abundant in 5	6/10	4/10
8	Absent from 4 smears, present (scanty) in 6	8/10	0/10
9	Absent from 3 smears, present (very scanty) in 7	1/10	0/10

E. coccoides was given as 0·2 ml. $\times 10^{-7}$ infected spleen suspension intraperitoneally. Filtrate was 20 hr. growth of *Salm. typhi-murium* (strain S_4) formolized (0·25 per cent) and filtered through a 540 mμ A.P.D. gradocol membrane. Dose was 0·2 ml. intraperitoneally.

none of them died. The next three groups (5, 6 and 7) showed a mortality of 5/10, 6/10 and 4/10. In group 8 the majority became ill but none died, and in the last group they remained well. Thus throughout the period of enhanced susceptibility, *E. coccoides* was plentiful in blood smears; the maximal enhancement occurred at about 1½–2 days after the parasite first appeared and mice recovered normal resistance to toxin with the disappearance of the parasite from blood smears. On the other hand, there appeared to be no consistently direct relationships between the recorded concentration of parasites in individual blood smears of mice of a group and their reactions to the toxic filtrate, and for this reason the results of individual blood smears have been omitted from Table III. From previous experiments the dose of filtrate used had been found sufficient to produce death in practically 100 per cent of mice in which *E. coccoides* had produced a state of maximum susceptibility to filtrate, yet the maximum mortality in this experiment was 6/10. This might suggest that the duration of maximal or nearly maximal susceptibility in relation to the time of injection of *E. coccoides* was less than 24 hr. and further experiments would be necessary to determine this.

In a further experiment to throw light on the action of *E. coccoides* in raising the susceptibility of mice to bacterial filtrate, 1 mg. of oxytetracycline in 5 per cent gum acacia was injected subcutaneously into groups of pre-treated mice at varying times before and after the inoculation with filtrate. None of 12 mice died which had been treated with oxytetracycline 12 hr. before they were inoculated with filtrate, whereas all of 10 mice died which had been treated 6 hr. before filtrate inoculation. Other groups of mice treated with oxytetracycline less than 6 hr. before filtrate inoculation and after filtrate inoculation and an untreated group all showed a high mortality. These results were elucidated by taking blood smears from the mice of a control group (not inoculated with filtrate) at the time of its injection with oxytetracycline and 2, 4 and 6 hr. afterwards. *E.*

coccoides was very abundant in the smears taken at the time of giving the drug and 2 hr. afterwards; at 4 hr. the number of *E. coccoides* in smears had become slightly less while at 6 hr. they had practically disappeared from the peripheral blood.*

Properties of the toxic filtrate

The toxic principle of 540 mμ A.P.D. gradocol membrane filtrate of formolized 20-hr. growth of S_4 resisted boiling for 15 min. and was non-dialysable. It was readily filtrable through a gradocol membrane of 200 mμ A.P.D. but not through one of 50 mμ A.P.D. After intravenous injection results were comparable with those obtained by the intraperitoneal route, whereas only 2 of 23 pre-treated mice died after subcutaneous inoculation with 0·2 ml. of filtrate. These results are based on two independent experiments which gave completely consistent results. In a single experiment toxic filtrate was titrated in groups of *E. coccoides* pre-treated mice aged 10–12 weeks and compared with mice of the age used in other experiments (3 weeks) with the following results :

Dose of filtrate (ml.)	Mortality ratio in mice aged	
	10–12 weeks	3 weeks
0·02 . . .	2/10	4/10
0·05 . . .	5/10	3/10
0·2 . . .	10/10	10/10

Older mice thus appear to be as susceptible as younger mice and the use of older mice for experiments of the kind described here might be preferable to the use of weanling mice.

Toxicity of filtrates of other organisms for pre-treated mice

Since the above results were obtained with a single virulent strain of *S. typhi-murium* isolated in this laboratory, experiments of the same kind were carried out with other bacteria. Culture filtrates from all strains of *S. typhi-murium* whether virulent or avirulent, were lethal to a high proportion of pre-treated mice. Filtrates from other species of *Salmonella* were also toxic and the mortality produced by all the species tested, including the strains of *S. typhi-murium* (other than S_4), was 30/53 or 57 per cent. With 4 strains of *Bact. coli* 12 of 25 pre-treated mice became ill (48 per cent) and 10 died (40 per cent). Similarly, with 4 strains of *Shigella* 16 of 32 mice became ill (50 per cent) and 5 died (16 per cent). In contrast, filtrates from growths of 3 species of Gram-positive organisms, *Str. pyogenes*, a coagulase-positive *S. aureus* and a virulent strain of *Ery. rhusiopathiae*, had no visible effect on pre-treated mice. Filtrates from both the Gram-negative and the Gram-positive organisms were non-toxic, or almost non-toxic, for normal mice.

Toxicity of lipopolysaccharides from Gram-negative organisms for pre-treated mice

It seemed possible that the toxic principle in culture filtrates of Gram-negative bacteria might be related to the endotoxins which can be extracted from these

* It should be noted that the VS strain of mice does not carry *E. coccoides*. Although the response to an injection of *E. coccoides* in mice which carry this parasite has not been fully investigated, probably it would be insufficient to render them highly susceptible to culture filtrate.

organisms. Since the endotoxins of Gram-negative bacteria are their somatic antigens and consist of lipopolysaccharides united to protein (Wilson and Miles, 1955), lipopolysaccharides derived from endotoxins might prove to be much more toxic for pre-treated mice than for normal mice. Through the courtesy of Dr. Rowley and his colleagues we obtained samples of lipopolysaccharide extracted from a strain of *Bact. coli* (strain CV) and we inoculated normal and pre-treated mice with varying doses. The results of experiments carried out on different occasions were variable in both pre-treated and normal mice; for example, in one experiment a dose of 50 μg. killed one of 8 normal mice and 100 μg. killed all of 6 mice; yet in a comparable experiment on another occasion, 80 μg. failed to kill any of 14 mice and 100 μg. failed to kill any of 6 mice. Preliminary experiments appear to suggest that such discrepancies are due to a marked variation of the toxicity of lipopolysaccharide and it was also found that environmental temperature affected the mortality rate of the mice; this possibility which might have important implications is being studied further. When doses of 10 μg. and even 2 μg. were injected intravenously into groups of 5 pre-treated mice they died within 24 hr. whereas doses of 10 μg. and 50 μg. failed to kill any mice when injected by the same route into groups of 5 normal mice. In another experiment, 10 μg. killed all of 6 pre-treated mice and 5 μg. killed two of 6, whereas 50 μg. killed only one of 8 normal mice. Similar results were obtained with lipopolysaccharide from *Pasteurella pseudotuberculosis* and with that from *Chromobacterium prodigiosum*. In order to obtain the ratio of the LD_{50} of lipopolysaccharide for normal mice to that for pre-treated mice, graduated doses of the *Bact. coli* lipopolysaccharide were injected intravenously or intraperitoneally into groups of normal and pre-treated mice. The ratios obtained in four such experiments were 11 : 1, 100 : 1, $>$ 11 : 1 and $>$ 20 : 1. The failure to obtain determinate ratios in the two latter experiments was due to failure to produce a lethal effect in normal mice even with the highest doses of lipopolysaccharide used. The results, however, indicate that the ratio probably lies between 10 : 1 and 100 : 1.

DISCUSSION

The experimental observations with lipopolysaccharides from Gram-negative bacteria support the hypothesis that the toxic principle present in young culture filtrates of such bacteria is endotoxin. By concentrating filtrates we hope to show that they are as toxic as the purified lipopolysaccharide. Preliminary experiments suggest that inoculation of normal mice with filtrate increases resistance to the combined treatment with *E. coccoides* and filtrate. Attempts to produce antiserum in rabbits which neutralizes the toxin have not yet been fully successful. Investigation along these lines is being continued.

We consider that pre-treatment of mice with *E. coccoides* might be a valuable aid to investigation of the rôle of endotoxins in the pathogenicity of the coli-typhoid group which might perhaps lead to a better understanding of some forms of food poisoning and infantile diarrhoea.

SUMMARY

Filtrates of 18–20 hour growths of the coli-typhoid and dysentery groups of bacteria were toxic for mice pre-treated with *E. coccoides* when injected intravenously or intraperitoneally but not subcutaneously. Some filtrates contained a

toxic material which was lethal, especially filtrates of *S. typhi-murium* and some other *Salmonellae*. Other filtrates produced manifest illness within a few hours with complete recovery within 24 hours. All the filtrates were almost non-toxic for normal mice. In contrast, filtrates of growths of a group A streptococcus, a strain of *Staphylococcus aureus* and a strain of *Erysipelothrix rhusiopathiae* were not toxic for pre-treated mice.

The toxic principle of the filtrate resisted boiling for at least 15 minutes and was not dialysable. It was easily filtrable through a gradocol membrane of average pore diameter 200 mμ but detectable amounts did not pass through a membrane of A.P.D. 50 mμ.

The lipopolysaccharides extracted from the endotoxins of *Bacterium coli*, *Pasteurella pseudotuberculosis* and *Chromobacterium prodigiosum* were lethal for *Eperythrozöon coccoides* pre-treated mice in much smaller doses than for normal mice.

It is suggested that the toxic principle of filtrates of growths of these Gram-negative organisms might be lipopolysaccharide.

We wish to record our thanks to Dr. Joan Taylor of the Central Public Health Laboratory for identifying our strain of *Salm. typhi-murium* and for supplying us with many cultures. We also wish to thank Dr. R. J. W. Rees of the National Institute for Medical Research for useful suggestions during the early stages of this work, Dr. D. Rowley and his colleagues of the Wright-Fleming Institute of Microbiology and Dr. J. H. Humphrey of the National Institute for Medical Research for the generous supply of bacterial lipopolysaccharides. Finally, we gratefully acknowledge the technical assistance of Mr. A. Lane, Miss E. Brodaty and Mr. D. Wood.

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THE CARCINOGENICTIY OF CERTAIN COMPOUNDS RELATED TO TRYPAN BLUE

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TRYPAN BLUE, Congo red and Trypan red have been tested for carcinogenic activity in the rat (Gillman and Gillman, 1949; Simpson, 1952). Of these compounds, only Trypan blue injected subcutaneously into rats at weekly or bi-weekly intervals induced reticulum cell sarcoma of the liver. The present investigation has been extended to the following compounds related to Trypan blue which have not been previously tested.

Sodium diphenyl-4,4′-*bis*-azo-(2″)-8″-amino-1″-naphthol-3″,6″-disulphonate (Direct blue BB) and sodium 2-methylbenzene-azo-(1,2′)-8′-amino-1′-naphthol-3′,6′-disulphonate (Semitrypan blue) failed to produce neoplastic changes in the livers of rats when injected subcutaneously in the groin at weekly or bi-weekly intervals, but 2 out of 10 rats stained with 160–200 mg. of the former compound developed sarcoma at the site of injection.

MATERIALS AND METHODS

The rats were bred in our laboratory. At the start of the experiment they were about 3–4 months old and weighed 100–159 g. Their food was a mixture of unpolished rice and greens. There were 12 males and 28 females, 6 and 14 of each being kept as one experimental group.

Doses of 1 ml. of a 1 per cent solution of Direct blue BB and of a 2 per cent solution of Semitrypan blue dissolved in cold sterile distilled water were injected subcutaneously into each experimental group at weekly or bi-weekly intervals. The injections were discontinued after the total dosage of Direct blue BB and Semitrypan blue amounted to 200 and 400 mg. respectively. The numbers of rats which were alive before the dye injections were stopped were 5 and 17 in the Direct blue BB and Semitrypan blue groups respectively.

The rats were allowed to die or were killed when death seemed imminent. At post-mortem examination pieces of liver were removed for histological examination in all cases and an extensive survey was made of the other organs, including lymph nodes, spleens and testes.

Preparation of the Dyes Injected

Raw materials used for the preparation

o-Toluidine.—Once redistilled reagent class *o*-toluidine was converted into *o*-acetotoluide. After recrystallization of the acetyl compound twice from water, it became sufficiently pure (m.p. 111°). It was then hydrolysed in an alkaline medium, and the recovered *o*-toluidine was redistilled again and the main distillate was corrected (b.p. 197–202°).

Benzidine.—c.p. reagent (m.p. 125–7°).

H acid (monosodium salt).—Technical grade H acid was precipitated once from sodium carbonate solution by hydrochloric acid, then recrystallized 4 times from water. It showed the appearance of white cotton linter and assayed to be 89 per cent pure.

Direct blue BB

The solution made from 4 ml. of conc. HCl, 60 ml. of water and 3·69 g. of benzidine was cooled to 10° and 4·2 ml. of conc. HCl was added. At this temperature 2·85 g. of sodium nitrite (purity : 97 per cent), *i.e.*, twice the amount equivalent to benzidine, in 10 ml. of water was added, and was maintained at 5–10° for 30 min.

Meantime, 15·35 g. of the purified H acid was dissolved in 100 ml. of water with the addition of 1·6 g. of NaOH to make the solution nearly neutral, then cooled to 10°, and 7 g. of sodium carbonate was added immediately before the coupling. The diazonium solution was added rather rapidly under vigorous stirring. After 30 min. the coupled solution was raised to 15° and stirred at this temperature for 4 hr., then at 50° for 30 min. Next day, the solution was warmed to 85°, and salted out by 12 g. of pure sodium chloride for each 100 ml. of the solution. After cooling, the separated dye was filtered. Then the salting out of the dye from hot solution by 10–12 per cent of sodium chloride and the filtration of the precipitated dye after cooling, were repeated 8 times. In the final step, before the addition of the salt, the hot solution was filtered. The final product, weight 22·2 g., was almost free from organic impurities and assayed to be 90·4 per cent pure, the admixture being only pure sodium chloride.

Semitrypan blue

To the solution of 13·7 ml. of 6 N-HCl in 9·0 ml. of water was dissolved 3·21 g. of the purified *o*-toluidine. The solution was chilled to 0°, and diazotized with an equivalent amount of sodium nitrite (purity : 98 per cent), *i.e.*, 2·11 g. of it were dissolved in 10 ml. of water. The diazotized solution was kept at 0–5° for 30 min., at the end of which time the presence of free nitrous acid was tested.

Meanwhile, a paste was made from 12·0 g. of the purified H acid and 75 ml. of water, then it was dissolved by the addition of 1·40 g. of NaOH in 5 ml. of water. The solution was chilled to 0°, and just before the coupling 7·3 g. of sodium carbonate was added. With vigorous agitation the diazonium solution was slowly added into the H acid solution. The solution was stirred at 10° for a total of 6 hr. Next day, the solution was warmed to 85° and the dye was salted out by the addition of 20 g. of powdered sodium chloride for each 100 ml. of the solution. The precipitated dye was dissolved in 350 ml. of warm water, 2 g. of decolorizing carbon were added, and the solution was filtered after it was stirred for 20 min. Then the salting out of the dye from hot solution and filtration of the salted cake were repeated 5 times more. In the final step, before the addition of the salt, the hot solution was filtered. After drying of the cake, the yield was 14·55 g. The final product was almost free from organic impurities.

RESULTS

Length of survival and tumour incidence are shown in the Table. By the 150th day 8 rats in the Direct blue BB group and 2 in the Semitrypan blue group had died and were free of tumours. At this time the remaining 12 and 18 rats in each group had received injections of 140 mg. of Direct blue BB and 300 mg. of Semitrypan blue respectively. Spindle cell sarcoma developed near the injection site in 2 of 10 rats injected with 150 mg. or more Direct blue BB, after the experimental period of 190–200 days. The first tumour caused death 211 days after starting the dye injection and the second after 216 days. No tumour occurred in the other rats.

Gross findings

Respiratory infections were frequent in rats of the Direct blue BB group. The majority of young adult rats lost weight during injections of the dye from the start of the experiment and their general appearance was bad throughout the total experimental period. Some gained weight slightly during the first 3 months, followed by a gradual decrease later. The last rat died from respiratory infection

TABLE.—*Length of Survival and Tumour Incidence in Rats Treated with Direct Blue BB and Semitrypan Blue*

Rats receiving 10 mg. Direct blue BB at weekly or bi-weekly intervals					Rats receiving 20 mg. Semitrypan blue at weekly or bi-weekly intervals				
Rat No.	Sex	Survival in days	Amounts of dye injected (mg.)	Tumour	Rat No.	Sex	Survival in days	Amounts of dye injected (mg.)	Tumour
1	F.	16	30	—	1	F.	50	140	—
2	,,	16	30	—	2	,,	67	180	—
3	,,	23	40	—	3	,,	197	360	—
4	,,	65	90	—	4	,,	271	400	—
5	M.	78	100	—	5	,,	300	400	—
6	,,	87	100	—	6	,,	300	400	—
7	,,	118	120	—	7	,,	300	400	—
8	F.	120	120	—	8	M.	300	400	—
9	,,	156	140	—	9	,,	300	400	—
10	,,	165	150	—	10	F.	300	400	—
11	,,	191	160	—	11	,,	300	400	—
12	,,	211	170	*	12	M.	300	400	—
13	,,	216	180	*	13	F.	300	400	—
14	M.	226	180	—	14	,,	300	400	—
15	F.	229	180	—	15	,,	300	400	—
16	M.	248	200	—	16	M.	300	400	—
17	F.	254	200	—	17	,,	300	400	—
18	M.	262	200	—	18	F.	300	400	—
19	F.	268	200	—	19	,,	300	400	—
20	,,	270	200	—	20	M.	300	400	—

* The presence of tumour was found at a period of 165–190 days after the start of the experiment.

after 270 days. On the other hand, animals on Semitrypan blue gained weight during the first 6 months, and thereafter, maintained their weight for about 4 months. Throughout the dye-injecting period of 7 months, the general appearance of the animals was good in all cases.

The livers at autopsy showed some degree of atrophy in rats of the Direct blue BB group, and were about half the size of those in the normal untreated rats and where no neoplastic changes were present. The liver findings with Semitrypan blue were similar to normal. The former livers were stained with the dye injected and they were blue in colour, but the latter contained no dye.

Two of the Direct blue BB group showed projections of the groin region injected where the tumours were present. The tumours presented themselves as solid nodules throughout the groin, measuring $5{\cdot}5 \times 4{\cdot}0 \times 3{\cdot}5$ cm. and $2{\cdot}5 \times 2{\cdot}5 \times 2{\cdot}5$ cm. No metastases were found. The other organs showed no significantly abnormal features.

Microscopy

Atrophy of the parenchymal cells and dilatation of sinusoid were seen in livers of the Direct blue BB group. Hyperplasia and hypertrophy of Kupffer's stellate cells or histiocytes in the portal tracts seen in Trypan blue treated rats were not noticed in the present series. In the kidney, proximal convoluted tubule cells showed considerable degeneration and blue pigmentation. In the spleen, some hyperplasia of histiocytes was found. Noticeable alterations were not found in the heart, pancreas, ovary and testis except for slight atrophic changes. The tumour nodules consisted of spindle-shaped cells for the most part with fewer

polymorphic or multinuclear giant cells, and apparent transitions between these cell types. Mitoses were not frequent in these cells. Abundant reticular fibres around individual tumour cells were demonstrated by silver impregnation and some collagen by van Gieson's stain. Histogenetically it is probable that this tumour is of fibroblastic origin and it is regarded as a fibrosarcoma.

In the Semitrypan blue group, slight vacuolation of parenchymal cells of the liver and hyperaemia of glomerular capillaries in the kidney were noticed. Heart, spleen, pancreas, ovary and testis appeared normal.

DISCUSSION

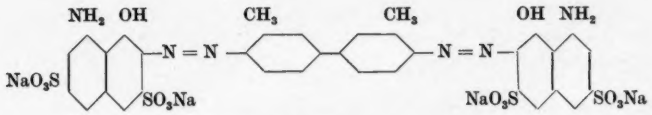
Many aminoazobenzene derivatives have been tested for carcinogenic activity in the rat. In 1945, Miller and Baumann found that N,N-dimethyl-3′-methyl-4-aminoazobenzene was more carcinogenic than the parent compound, N,N-dimethyl-*p*-aminoazobenzene. Thus, introduction of a methyl group in the 3′ position may change the character of the parent carcinogen into a more powerful carcinogenic compound. But regarding the tumours induced by these two dyes, there was no difference noted on the basis of histogenesis and histological criteria.

Gillman and Gillman (1949), and Simpson (1952) reported reticulum cell sarcoma in the livers of rats stained for a long period with Trypan blue. Trypan blue is sodium 3,3′-dimethyldiphenyl-4,4′-*bis*-azo-(2″)-8″-amino-1″-naphthol-3″, 6″-disulphonate. Regarding the cause of the malignancy, Simpson pointed out two possibilities, *viz.*, one in which Trypan blue is itself a carcinogen and the other in which the dye in the liver slowly liberated a carcinogen, probably 2,8-diamino-1-naphthol-3,6-disulphonic acid or sodium 4′-amino-3,3′-dimethyl-diphenyl-azo-(4,2″)-8″-amino-1″-naphthol-3″,6″-disulphonate, over a long period (Fig. 5).

Semitrypan blue is a red dye in distilled water. When this dye was injected subcutaneously into rats, the body appeared coloured red for about 10 hours. But after 1 hour, the dye began to be excreted in the urine, until at a stage 1 day after the injection the rats lost all traces of red colour. On the other hand, rats which received injections of Direct blue BB were vitally stained for over 2 weeks with the dye. From these facts and the production of fibrosarcomas at the site of injection in 2 out of 10 rats stained for 6–7 months with Direct blue BB and no tumour by Semitrypan blue injections, vital staining with the dye might account for one of the first steps in the carcinogenic process, though known vital staining dyes are not always found to be carcinogenic. Thus, the concentration of the dye in the normal tissue from which tumour arises and the presence of the dye itself or the slow liberation of a carcinogenic derivative over a long period may be responsible for the tumour formations (Simpson).

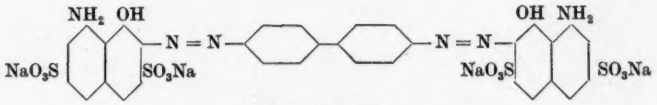
Both Trypan blue and Direct blue BB were taken up by the reticulo-endothelial system. And it is theoretically possible that reduction of the diazo group *in vivo*, similar to Trypan blue, occurs in the conversion of Direct blue BB to 2,8-diamino-1-naphthol-3,6-disulphonic acid and benzidine. There is no evidence that benzidine and *o*-toluidine have any carcinogenic properties. Hence, if the former derivative, as pointed out by Simpson, is the primary carcinogen for tumour formation induced by Trypan blue, rats on Direct blue BB might be expected to develop reticulum cell sarcoma. But no neoplastic changes in livers were seen in the present series of rats which received injections of Direct blue BB, and tumours which might show different growth characteristics appeared at the site

of injection. The methyl groups in the 3 and 3′ positions of *o*-toluidine might account for some of the differences. The question whether sodium 4′-amino-3,3′-dimethyldiphenyl-azo-(4, 2″)-8″-amino-1″-naphthol-3″, 6″-disulphonate is the cause of reticulum cell sarcoma formation therefore remains an open one.



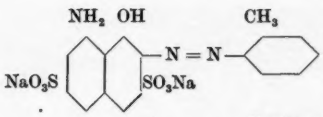
Trypan blue.

Sodium 3,3′-dimethyldiphenyl-4,4′-*bis*-azo-(2″)-8″-amino-1″-naphthol-3″,6″-disulphonate.



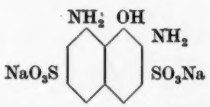
Direct blue BB.

Sodium diphenyl-4,4′-*bis*-azo-(2″)-8″-amino-1″-naphthol-3″,6″-disulphonate.

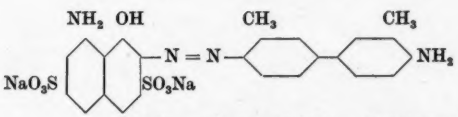


Semitrypan blue.

Sodium 2-methylbenzene-azo-(1,2′)-8′-amino-1′-naphthol-3′,6′-disulphonate.



2,8-diamino-1-naphthol-3,6-disulphonic acid.



Sodium 4′-amino-3,3′-dimethyldiphenyl-azo-(4,2″)-8″-amino-1″-naphthol-3″,6″-disulphonate.

FIG. 5.—Structural formulae of the dyes discussed.

SUMMARY

Sodium diphenyl-4,4′-*bis*-azo-(2″)-8″-amino-1″-naphthol-3″,6″-disulphonate (Direct blue BB) and sodium 2-methylbenzene-azo-(1,2′)-8′-amino-1′-naphthol-3′,6′-disulphonate (Semitrypan blue) were prepared in our laboratory to determine whether Trypan blue-induced tumours of rats were produced by related compounds.

The present series of rats receiving 20 injections of 10 mg. Direct blue BB and of 20 mg. Semitrypan blue for over 300 days showed no neoplastic changes in the livers.

Fibrosarcomas occurred at the site of injection in 2 of 10 rats surviving 165–200 days of staining with Direct blue BB. The minimum carcinogenic dose was 150 mg. of dye.

There still remains the question as to which component of the dye is responsible for the difference in malignancy.

Our thanks are due to Prof. K. Hotta, Prof. F. Oshima and Dr. Y. Ushijima for their encouragement and assistance. Mr. Matsuyama prepared the sections and took the photomicrographs.

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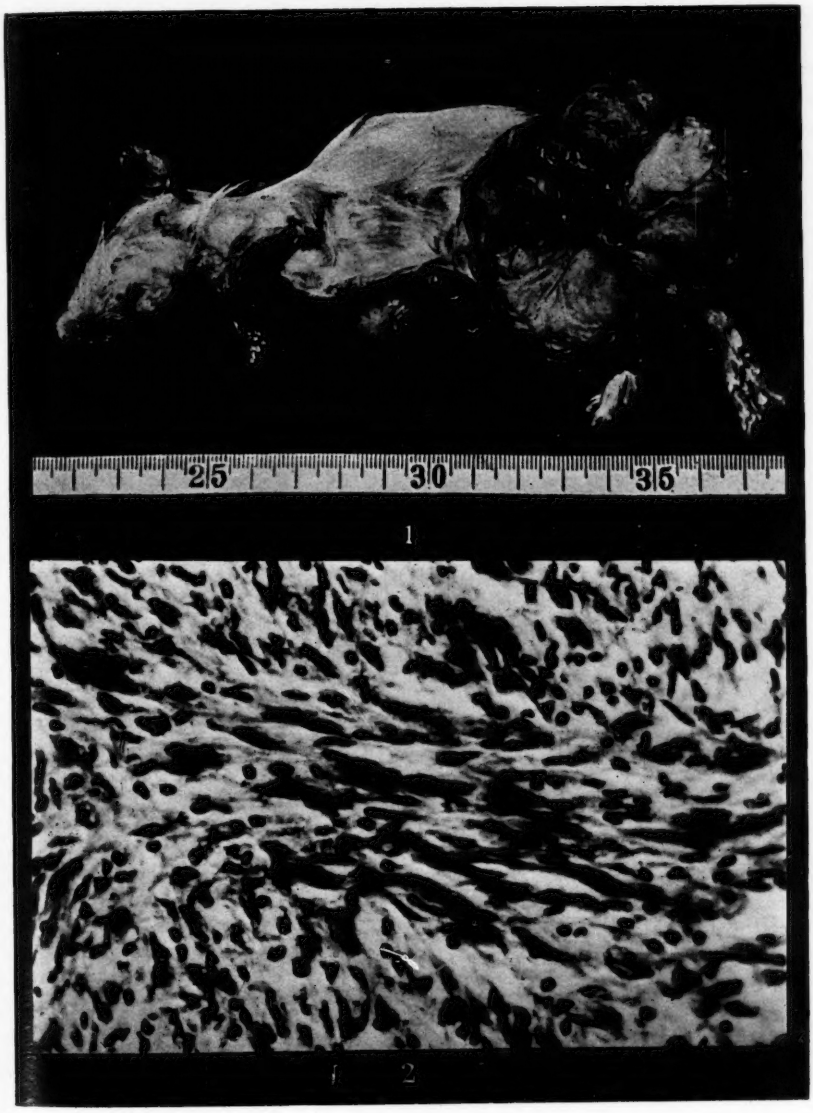
EXPLANATION OF PLATES

Photographs of tumours produced in rat No. 12 (Table).

FIG. 1.—Gross appearance.
FIG. 2.—Area showing spindle-shaped cells. H. & E. × 400.
FIG. 3.—Area showing pleomorphic and giant cells. H. & E. × 400.
Fig. 4.—Extensive reticulin formation. Silver impregnation. × 400.



Fujita, Mine, Iwase, Mizuno, Takayanagi, Sugiyama and Arai.

THE EFFECTS OF INTENSIVE AND PROLONGED ADMINISTRATION OF IRON PARENTERALLY IN ANIMALS

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THE problem of the relationship of haemosiderosis to haemochromatosis has not yet been solved, although in recent years it has assumed increasing importance in view of the growing numbers of reported cases of so-called " post-transfusional " or " exogenous " haemochromatosis. Clinicians are sharply divided on this subject. It is thus a fit one for experimental study, always bearing in mind the limitations of such an approach. The consequences of the introduction of excessive quantities of iron in various forms into the living animal have been the subject of repeated investigations (Polson, 1928, 1929, 1933 ; Cappell, 1930 ; Brown, Moore, Reynafarje and Smith, 1950 ; Brown, Smith, Dubach, Reynafarje and Moore, 1956 ; Nissim, 1953 *a*, *b* ; Pinniger and Hutt, 1956 ; Moore and Dubach, 1956). Each succeeding study has served to illustrate more clearly that exogenous iron in colloidal complex form is treated by the animal body and distributed within it in a manner which differs conspicuously from that characteristic of human idiopathic haemochromatosis (Golberg, 1957). With the advent of an iron-dextran complex possessing unique properties with regard to pH stability, low toxicity and absorption from muscle (Martin *et al.*, 1955), the opportunity arose of undertaking a fresh study of the problem of exogenous siderosis.

Our objectives in carrying out this work were as follows : firstly, to examine the effects of doses of iron greater than had been possible hitherto ; secondly, to study the cycles of redistribution of the iron from its primary sites of deposition ; thirdly, to assess the function of the organs principally affected by siderosis, particularly the liver ; and lastly, to trace, as far as possible, the influence of the dextran moiety on the behaviour of the complex as a whole.

MATERIAL AND METHODS

Animals and dosage

The experiments were carried out on young albino rats weighing for the most part 110–140 g. and fully-grown rabbits of various breeds weighing approximately 2–3 kg. Equal numbers of each sex were present in each group.

Injections of iron-dextran (" Imferon ") solution containing 50 mg. of elemental Fe/ml. were given into the muscles of the hind limbs, alternating from one side to the other and attempting, so far as possible, to vary the site at which the solution was deposited. Dose schedules of iron-dextran are given in Table I. For each 1 ml./kg. of iron-dextran given to the corresponding test animals, the control animals were given 1 ml./kg. of 20 per cent dextran solution similar to that employed in the production of the iron-dextran complex.

In addition to the high dosage groups listed, there were numerous other groups of rats and rabbits on lower doses, approximating at the lower levels to those customary in clinical practice, namely a total of about 20 mg. Fe/kg. Detailed reference will not be made to these groups, since the lesions to be described did not appear in them. It is of interest, however, that within these groups were animals which had had up to 53 injections of iron-dextran with no apparent untoward effect.

TABLE I.—*Experimental Details*

Species	Group	Dose* (mg. Fe/kg.)	Number of injections	Total dose (mg. Fe/kg.)	Number of animals: Iron-dextran	Number of animals: Dextran control	Times of sacrifice (weeks)†
Rats	A	75	22	1650	38	12	9–60
	B	75	12	900	12	6	4–50
	C	25	36	900	13	4	18–55
	D	25	24	600	12	4	12–48
	E	25	12	300	12	4	7–49
Rabbits	—	75	11	825	26	16	2–59

* Frequency of injections: 75 mg. Fe—three times weekly; 25 mg. Fe—twice weekly.

† Times of sacrifice are expressed in weeks after the start of injections. Test and control animals were killed at intervals throughout these periods.

Investigations

The animals were weighed weekly. The following investigations were carried out: (*a*) During the life of the animals: routine haematological examination of the blood including haemoglobin, haematocrit, erythrocyte sedimentation rate, leucocyte count, differential count and stained smear; hepatic and renal function tests, oral and intravenous glucose tolerance tests; electrophoresis of urinary proteins, urinary amino acid chromatography, urinary 17-ketosteroid excretion; examination of seminal fluid, including sperm count, motility, respiration, fructolysis index.

(*b*) Post mortem: liver, spleen, heart, kidneys, adrenals and gonads were weighed; specimens of liver, spleen, kidney, bile and serum were taken for estimation of their iron content; 43 blocks were taken for histological examination. In a few animals respiration studies were carried out on liver, kidney and heart.

Standard methods were used throughout. Details of some are given below, in reporting the results; others were referred to in a previous publication (Beresford, Golberg and Smith, 1957). For the determination of neutral 17-ketosteroids, urine was collected over 72 hr. from rabbits or pairs of rats. Extraction and final measurement was carried out by the method of the Medical Research Committee in Clinical Endocrinology (1951), going through the Girard P derivatives prepared according to Talbot, Butler and Maclachlan (1940).

The procedure at autopsy was to anaesthetize the animal with ether, expose a carotid artery and exsanguinate the animal while taking due precautions to avoid haemolysis in the blood being collected. In view of the large amounts of iron stored in organs like the liver, it was considered unnecessary to carry out perfusion prior to chemical analysis.

RESULTS

Growth and Well-being of the Animals

Rats tolerated the injections very well. The only obvious adverse effect was impairment of growth at the higher dose levels, which was severe only in male rats. The weight curves for group A (see Table I) rats are shown in Fig. 1; those of groups B, C and D were similar. Seven of the rabbits injected with iron-dextran died during the first 5 weeks; the survivors remained well and did not lose weight. There were no deaths among the dextran control rabbits, which maintained their weight very well. The significance of these early deaths among the treated rabbits is discussed below.

General Observations

Serum iron determinations carried out while the course of injections was still in progress revealed high levels of total iron in both the iron-treated rats and rabbits. After the injections ceased the serum iron levels returned to normal in a few weeks. The values in dextran control animals were in the normal range:

0·225–0·313 mg./100 ml. in rats and 0·113–0·287 mg./100 ml. in rabbits. There was a striking difference between the serum iron levels attained in the two species. For rats the values (in mg. Fe/100 ml.) did not exceed 1·95 while in rabbits the mean value was 57·2, with a range of 11·8–118·2. At the time, no systematic effort was made to follow the changes in serum iron concentration throughout the course of the injections. In view of these differences, however, a special study has since been made which not only confirms the more rapid uptake

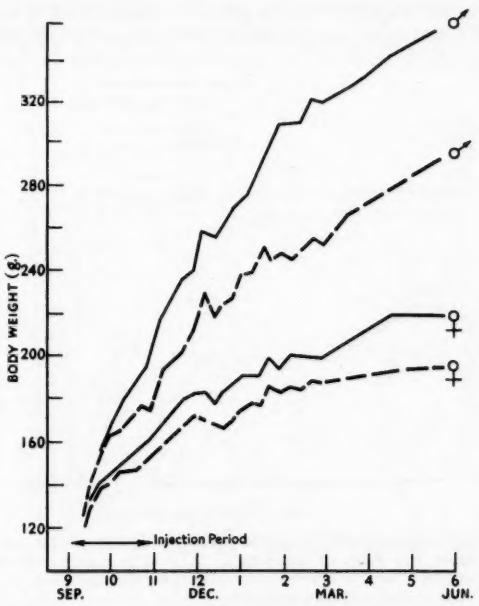


FIG. 1.—Growth curves of iron-treated and dextran control rats of group A. Thin continuous lines represent iron-treated animals and heavy interrupted lines the controls.

of iron-dextran from the circulation of the rat but also reveals an earlier absorption from muscle in this species. These results will be reported in detail elsewhere. In the rats grossly elevated serum iron concentrations were not reflected in a rise in urinary iron excretion, which at no time exceeded 0·012 mg. iron in 24 hr. and was usually 0·003–0·006 mg. In rabbits there was a considerable rise (see below).

Among the rabbits killed at 57–68 weeks, the lowest haemoglobin was 12·5 g./100 ml.; other indices were normal. The rats of groups A and B at 42 weeks and at 50–60 weeks had haemoglobin values of 14·7 g./100 ml. or over. Again red and white cell counts were within the normal range.

At autopsy in the test animals, there was obvious siderosis of the liver, spleen, inguinal lymph nodes and occasionally of lymph nodes elsewhere. The animals showed no naked-eye evidence of disease, apart from occasional bronchopneumonia in the rats. A special feature of the rabbit kidneys will be mentioned later. Brown staining of the subcutaneous tissues and muscles at the sites of injection

was the rule among the animals given high doses of iron-dextran. Other organs did not appear to be affected. No excess of fluid was found in the thoracic or abdominal cavities.

Observations on the Liver

Weight and iron content

The liver weight, expressed as a percentage of body weight, was compared in iron-treated and dextran control animals. It is clear from Fig. 2 that hepatomegaly is present in both species but is greater in iron-loaded rats, especially those on high dosage. In both species the effect follows rapidly on the start of injections.

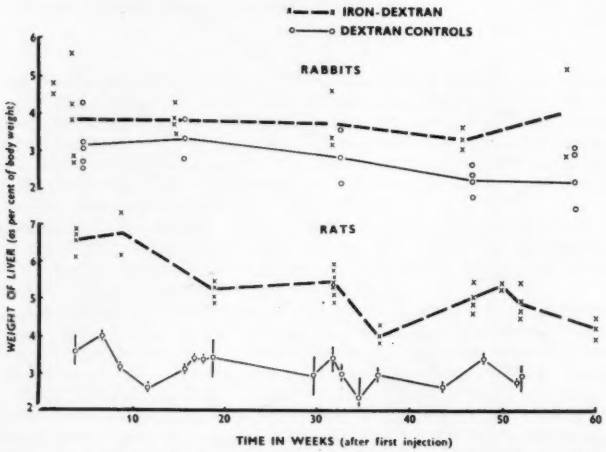


FIG. 2.—Ratio of liver weight to body weight in treated and control rats and rabbits, measured at various times after the start of injections. The thin continuous lines represent the dextran-treated controls; heavy interrupted lines connect the mean values for the group given the highest dose of iron-dextran complex.

The relationship between dose of iron-dextran administered and total iron content of each liver is shown in Fig. 3. The iron stored in the liver increases dramatically with the dose of iron-dextran. Nevertheless, the increased weight of the liver is attributable neither to the iron nor to storage of dextran (since chemical analysis and staining by alcoholic P.A.S. fail to detect such storage), nor to increased water content (mean percentage moisture content of liver: rabbits—iron-treated $73{\cdot}2 \pm 3{\cdot}6$, dextran controls $72{\cdot}9 \pm 1{\cdot}3$; rats—treated $71{\cdot}7 \pm 3{\cdot}5$, controls $71{\cdot}8 \pm 2{\cdot}3$). The dextran control animals show no change in liver weight as compared with untreated animals. Dextran storage in the liver is unlikely because of the low molecular weight of the polymer used in making iron-dextran complex.

It is instructive to compare the liver iron with the total iron in the spleen; this also rises sharply with the dose of iron-dextran. During the second half of the experiment the liver iron did not appear to increase. In the spleen particularly on the highest doses, there was a distinct increase during this time (Fig. 3). The additional iron could have been derived from the liver without

having a detectable effect on the hepatic iron content. There is normally a tendency for the spleen of the rat to increase its iron content with aging and to retain haemopoietic activity, but neither of these factors can adequately account for the large rise in splenic iron observed in this instance.

Hepatic function

The hepatomegaly was not accompanied by any detectable disturbance of function in either rats or rabbits. Before the commencement of injections some rabbits showed a retention of bromsulphthalein (1–3 mg. at 15 min.) which is in excess of the normal values established by us, 0·41 ± 0·09 mg./100 ml. plasma

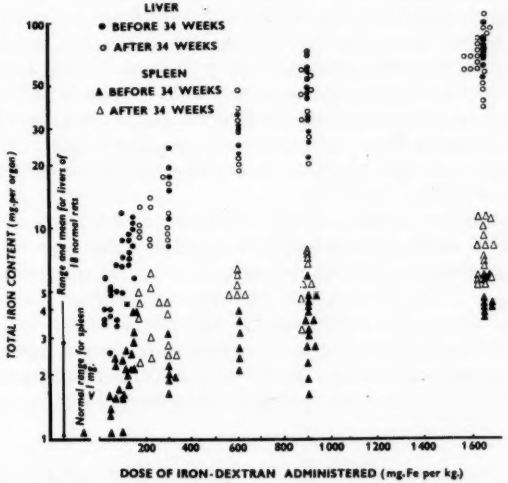


FIG. 3.—Iron content of rat liver and spleen in relation to the dose of iron-dextran administered (the scale of ordinates is logarithmic). Values for liver are represented by circles and for spleen by triangles. These symbols are solid for measurements carried out before 34 weeks after injection and open for values after 34 weeks

(Golberg and Smith, 1954). With the progress of the experiment these values did not deteriorate—some even returned to normal—and none exceeded 1·0 mg./100 ml. Other tests were normal with the exception of those for serum proteins. These were normal in the rat but in the rabbit their level fell to 3·5–4·6 g./100 ml. as proteinuria developed (see below). In the survivors the serum proteins returned to normal.

Tests of intravenous galactose and oral glucose tolerance were normal. Administration of glucose intravenously to iron-treated rabbits consistently gave rise to a flat blood glucose curve. Excretion of iron in the bile was not studied systematically but isolated analyses of gall-bladder bile gave values ranging from 0·2 to 10·4 mg. iron/100 ml. for treated rabbits and 0·13 to 0·25 mg. for dextran controls. It is probable that some of this iron is excreted and represents a mechanism for iron loss, albeit of limited capacity (Jasinski and Roth, 1954).

Histological changes in the liver

No parenchymal damage or abnormality other than siderosis is seen in the liver at any stage of the experiments. The pattern of deposition of iron, however, is different in the two species. In *rabbits* at the end of the injection period, the liver shows heavy deposition of iron in the parenchymal cells throughout the whole lobule with little iron in the Kupffer cells (Fig. 4); 3 months later parenchymal iron is reduced but there is more iron in Kupffer cells, some of which have become multinucleated giant cells (Fig. 5). This redistribution of iron is more striking at 6 months (Fig. 6), and by 9 months (Fig. 7) almost all the iron is found in Kupffer cells. At this time most of the siderophages have migrated into clusters around the central veins and in the portal tracts where they have stimulated a slight deposition of reticulin and collagen. No further change is seen at 12 months and there is no evidence of incipient cirrhosis.

In *rats* immediately after the injections, iron is present chiefly in the Kupffer cells, many of which are enlarged, and little is seen in the hepatic parenchyma (Fig. 8). In the following months iron increases in the Kupffer cells and multinucleated siderophages are formed, similar to those seen in the rabbit; these tend to migrate towards the central veins and portal tracts but stimulate little reaction. Parenchymal iron appears gradually and by the end of 12 months large amounts are present (Fig. 9 and 10).

A feature of the rat liver is the appearance of a ceroid-like pigment. This is first seen in the large siderophages 3 months after the injection period, as rounded or oval, sharply defined masses 5–20 μ in diameter, staining bright orange with eosin. This ceroid-like pigment increases in later months, often assuming bizarre forms. It stains very intensely for iron but is negative with Mallory's basic fuchsin, Ziehl-Neelsen's stain, chrome haemalum, periodic acid-Schiff and with acetylated Sudan Black B; it does not fluoresce in ultraviolet light. All these reactions are positive with other smaller inclusions in the siderophages.

Observations on the Kidney

Weights and iron content

There was a striking contrast between rat and rabbit in regard to renal storage of iron (Table II). The rabbit kidney doubled its iron content on a total dose of 50 mg. Fe/kg. At 825 mg. Fe/kg. it had increased over twenty times by the end

TABLE II.—*Iron Content of Rat and Rabbit Kidneys, Expressed in mg./g. Fresh Tissue*

Species	Dose (mg. Fe/kg.)	Time after first injection (weeks)	Number of estimations	Iron content	
				Mean	S.D.
Rabbit	Untreated	—	12	0·089	0·025
	Dextran	4–39	7	0·075	0·022
	50	4–36	12	0·183	0·046
	825	4–16	8	2·010	0·316
	825	32–59	8	0·910	0·360
Rat	Untreated	—	10	0·095	0·045
	125–600	21–48	14	0·294	0·064
	900	4–51	12	0·422	0·154
	1650	34–52	13	0·476	0·165

of the course of injections and in the 3 months which followed. After a further interval of 4 months it was found that the level of iron was falling and at 1 year it had reached levels comparable with those of the rat kidney. Iron storage in the latter was far less intense, even on twice as high a dose of iron-dextran. The rat renal iron concentration appeared not to undergo any change with time.

Renal function

In the rabbits, within a week or two of starting the intramuscular injections of iron-dextran complex, massive proteinuria developed. On electrophoresis the urinary protein was found to be a mixture of albumin and globulins. From initial values of over 7 g./100 ml., the serum protein levels became reduced to 3·5 g./100 ml. by the 3rd week of injections; the most striking reduction was observed in the albumin level. Many of the animals showed gross oedema and seven died during the injection period. In the survivors the proteinuria rapidly diminished and by the 9th week only a trace of protein remained in the urine. By the 16th week the serum proteins were restored to approximately normal levels. Only one dextran control animal showed proteinuria and then only on a single occasion. In rats an abnormal urinary excretion of protein never occurred.

The range of blood urea levels (in mg./100 ml.) in the rats was between 18 and 60; in the surviving rabbits it was 51–65 at 12 weeks. At 46 weeks it was 30–65, and, for one rabbit, 97. In contrast to the normal urea concentration tests of the other animals, this rabbit showed poor concentrating ability. Its kidney revealed extensive scarring and round cell infiltration with evidence of tubular regeneration including mitoses.

Urinary iron excretion rose to high levels (0·37–1·44 mg./24 hr.) in rabbits during the period of massive iron injections but by 48 weeks it had fallen to the normal level of less than 0·1 mg./24 hr. There was no such increase in rats; animals of all treated and control groups maintained an output of urinary iron below 0·01 mg./24 hr.

Histological changes in the rabbit kidney

The kidneys of the 7 rabbits which died and of one which was killed during the period of proteinuria were large and pale. Histologically they show, in some degree, dilatation of Bowman's capsules and of tubules, particularly the distal convoluted tubules. The appearance closely resembles that of thyroid tissue (Fig. 11 and 12). There is very heavy deposition of iron in the glomerular capillary endothelium (Fig. 13) and small amounts are seen in the stroma and in some tubular cells. In addition, in 4 of these animals, there is thrombosis of the glomerular capillaries (Fig. 14): the thrombus is heavily laden with iron. Thrombosis of veins also occurred in 2 animals. Of 4 animals killed within two weeks of completing the injections, 2 have normal kidneys and 2 show a little dilatation of some of the Bowman's capsules and of isolated groups of distal tubules but no eosinophilic coagulum. The kidneys of 4 control animals killed at this time are normal. In the kidneys of rabbits killed 3, 6, 9 and 12 months after the injection period only minor cortical scars are found, with equal frequency in treated and control groups. The only rabbit showing other abnormality has been mentioned above. With time, iron rapidly diminishes in the glomerular capillary endothelium and at 12 months is almost undetectable (Fig. 15). Small amounts persist in the tubules and stroma.

Discussion (on rabbit kidney)

The complete stability of the iron-dextran complex in plasma would be expected to prevent intravascular precipitation. Certainly at low and medium doses the iron-dextran complex causes no renal damage. At the heaviest doses, however, renal lesions constitute the only functionally significant effects which have been encountered.

When saccharated oxide of iron is administered in large doses intravenously to rabbits, glomerular lesions and a nephrotic syndrome result (Ellis, 1956) which closely resemble the changes we have encountered. What is probably the same change has been briefly noted by Pinniger and Hutt (1956). Ellis demonstrated the part played in the pathogenesis of the lesions by the instability of the saccharated oxide at the pH of plasma, which results in the formation of precipitates within the glomerular capillaries. He was at a loss to account for the glomerular capillary damage which leads to proteinuria and thought it might be attributable to anoxia. It seems to us that one need look no further than to the heavy deposition amounting almost to incrustations of iron within the endothelial cells of the glomerular capillary walls. This may be occasioned by the effect of further concentration at the glomerulus of an already astronomically high serum iron level. The damage to endothelium at this site may be severe enough to lead to thrombosis: if less severe, proteinuria results. Over a period of weeks, iron disappears completely from the glomeruli, the functional integrity of the glomerular capillary endothelium is restored, and protein loss ceases.

Histological changes in the rat kidney

In the rat an entirely different renal " lesion ", which is a post-mortem artefact, develops. First seen in animals of group A killed at 9 weeks, *i.e.*, 2 weeks after

EXPLANATION OF PLATES

FIG. 4–7.—Livers of rabbits killed at varying times after injection of 825 mg./kg. of iron.

FIG. 4.—After 2 weeks. Heavy iron in parenchymal cells but little in the Kupffer cells. Prussian Blue. × 140.

FIG. 5.—After 12 weeks. More iron is present in Kupffer cells and less in the parenchyma. Many Kupffer cells are multinucleated syncytial siderophages. Prussian Blue. × 140.

FIG. 6.—After 26 weeks. The siderophages are larger and more numerous. Prussian Blue. × 140.

FIG. 7.—After 40 weeks. The siderophages have migrated to the portal tracts and central veins. Very little iron remains in parenchymal cells. Prussian Blue. × 35.

FIG. 8–10.—Livers of rats killed at varying times after the injection of 1650 mg./kg. of iron.

FIG. 8.—After 2 weeks. Iron is almost entirely confined within Kupffer cells some of which are enlarged. Prussian Blue. × 140.

FIG. 9.—After 12 weeks. The Kupffer cells have become multinucleated syncytial siderophages. No parenchymal iron is seen. Prussian Blue. × 140.

FIG. 10.—After 40 weeks. The siderophages are showing a tendency to migrate towards central veins and portal tracts. Parenchymal iron is now heavy. Prussian Blue. × 140.

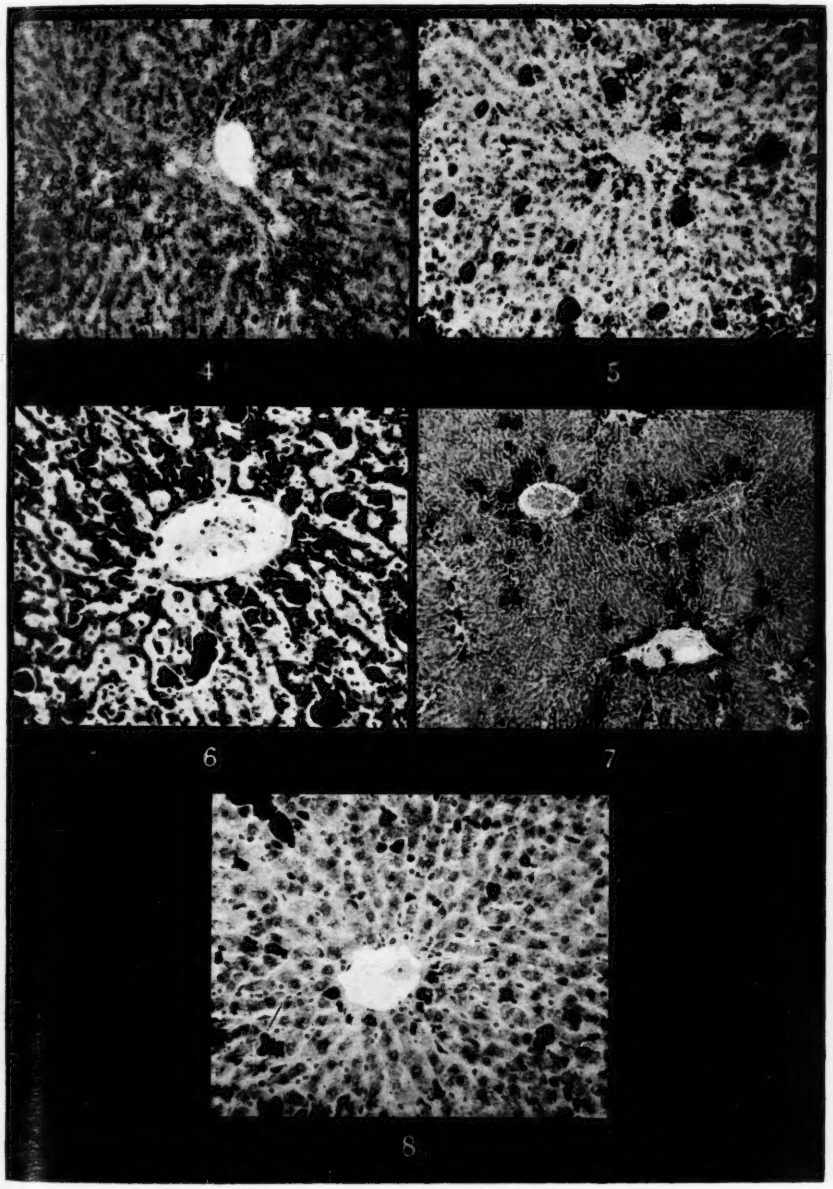
FIG. 11 and 12.—Kidney from rabbit with proteinuria which died during the injection period. Dilatation of Bowman's capsules and of tubules with coagulum in both. H. & E. × 140.

FIG. 13.—Heavy iron deposition in glomerular capillary endothelium seen during and for a short time after the injections. Prussian Blue. × 420.

FIG. 14.—Thrombosis of afferent arteriole and of glomerular capillaries in a rabbit which died during the injection period. Picro-Mallory × 240.

FIG. 15.—Complete disappearance of iron from glomerular capillaries but persistence of small amounts in the tubular epithelium from a rabbit killed 9 months after the injections. Prussian Blue. × 190.

FIG. 16.—Renal lesion in the rat. Apparent complete necrosis of epithelial cells of proximal convoluted tubules but with no cellular reaction in the tissues. P.A.S. × 450.



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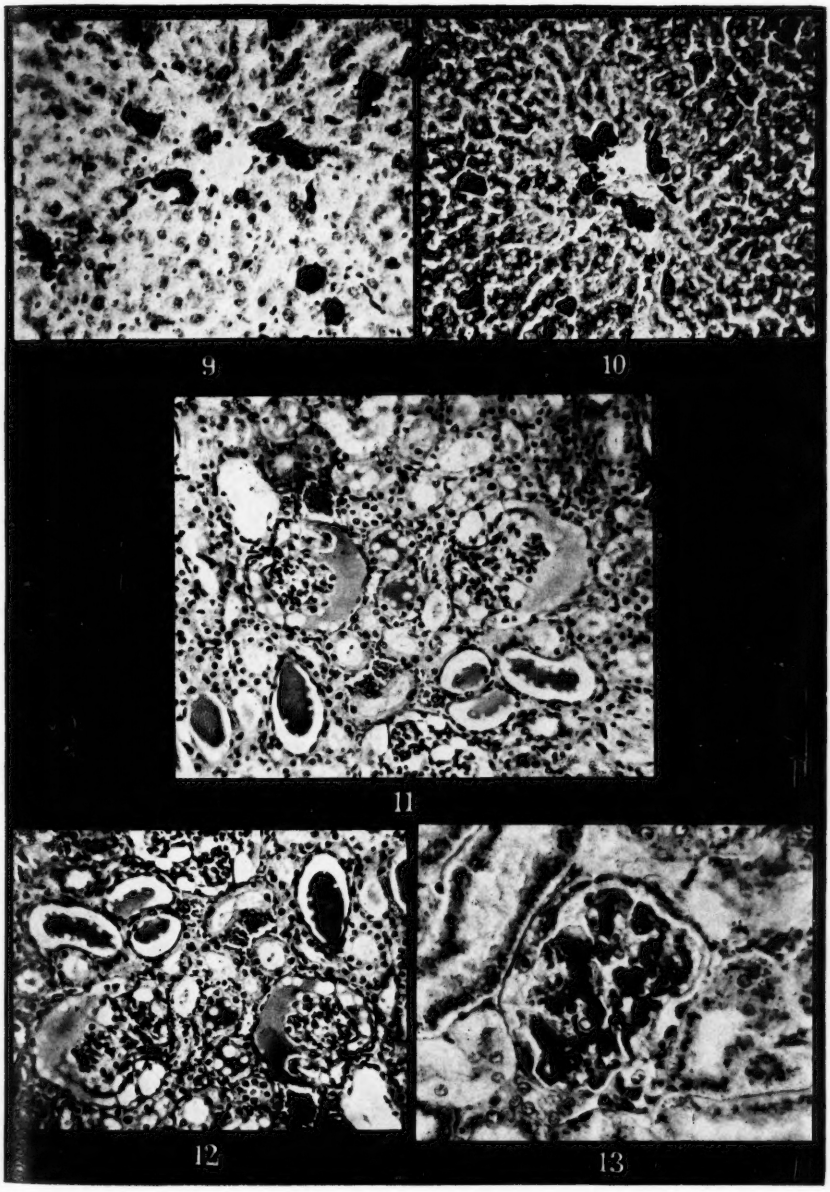

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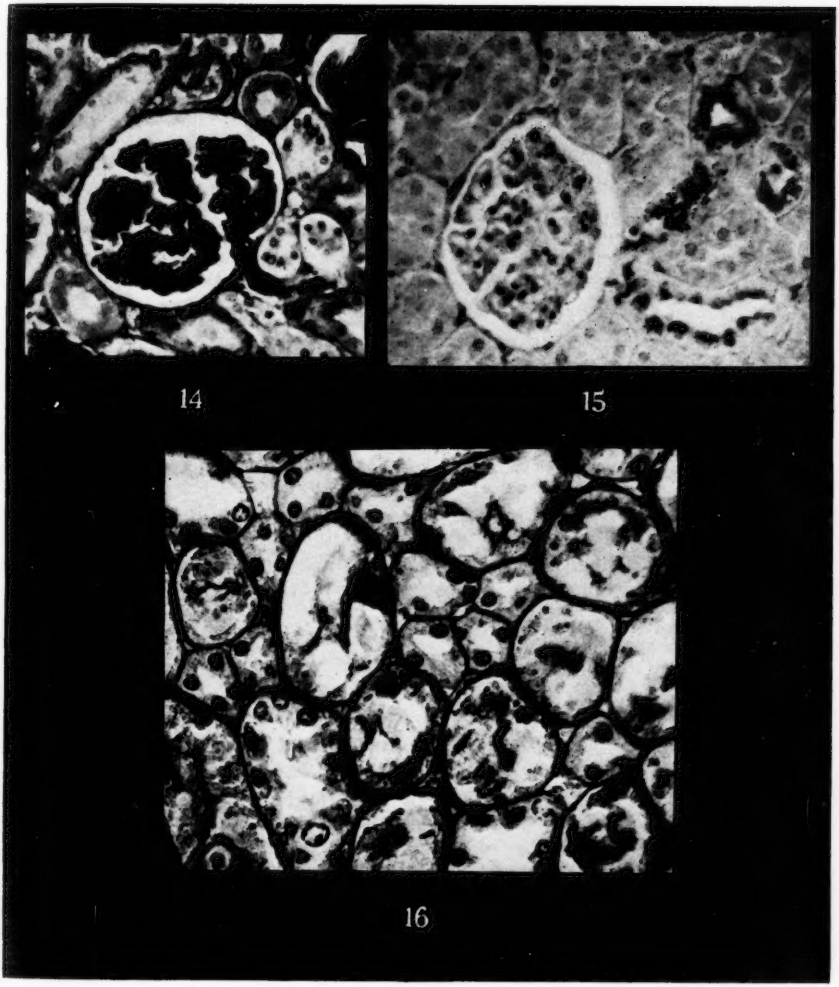

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ending the injections, the " lesion " consists of apparent necrosis (karyolysis and cytolysis) of the epithelial cells of most proximal convoluted tubules (Fig. 16). There is however no cellular reaction. Iron is very scanty in the glomeruli at all times but moderate amounts are seen in some of both healthy and necrotic proximal tubules. In the lumen of many tubules, chiefly necrotic ones, are rounded globules of a ceroid-like pigment which, unlike that in the liver, stains with Mallory's basic fuchsin, Ziehl-Neelsen's and periodic acid-Schiff stains, and fluoresces faintly in ultraviolet light. It does not contain iron. Much more of this pigment is found in animals killed later in the experiment but with time there is little variation in the amount of iron in the tubular epithelium.

Discussion (on rat kidney)

This apparently acute necrosis of renal tubules without cellular reaction in perfectly healthy animals seems a paradox. On further investigation it appears to be a post-mortem artefact of an unusual kind. Prompt fixation of the kidney within 15 min. of the death of the animal results in a completely normal picture, whereas a delay of 2–3 hr. in fixing the tissue leads to an extensive necrosis of the tubules. A detailed analysis of this phenomenon, which appears to be an acceleration of autolysis, will be published later.

Increased urinary iron excretion in mice has been stated by Chapelle, Gabrio, Stevens and Finch (1955) to be proportional to total body iron rather than to serum iron. Their studies included groups of mice treated with turpentine and phenylhydrazine. If these groups had been omitted, it is possible that in the iron-treated animals a relationship might have been demonstrable between urinary iron and serum iron levels. In the present experiments, the substantial rise in urinary iron in rabbits was associated with renal damage, which was not present in the rat. The state of the mouse kidneys was not reported by Chapelle and co-workers.

Observations on the Testis

Testicular function has been studied in detail at 12 and 24 weeks after the start of injections. There was a close correspondence between the characteristics of the seminal fluid in iron-loaded and dextran control rabbits. When these groups of rabbits, and corresponding groups of rats, were mated with normal untreated females the percentage fertility and the average size of litter was found to have remained unaffected by the heavy doses of iron-dextran administered to the male test animals.

The normal testes of control rabbits and rats show small amounts of iron in a few interstitial cells. In all the test animals there was interstitial cell hyperplasia. Interstitial cell iron increased considerably and did not appear to decrease with time. Spermatogenesis was unimpaired in all except two rats of group A killed one year after the injections began. In these animals the testes were shrunken and spermatogenesis was absent. The seminiferous tubules were small, empty, and lined only by a single layer of cells ; the interstitial cells were more numerous and contained much iron. Other rats of the same group killed at the same time, or of other (lower dose) groups, showed no such testicular changes.

Measurements of neutral 17-ketosteroid output suggest that a considerable increase occurs during and shortly after the period of injections. During the interval 4–15 weeks from the start of injections the results, expressed as mg./72 hr.,

were as follows: dextran control rabbits 0·42–2·03, mean 1·18; iron-loaded rabbits 0·80–4·95, mean 2·07. In rats the corresponding figures were: untreated rats 0·07; iron-loaded rats, 0·04–0·29, mean 0·14. The increase was particularly noticeable in rabbits at 4–7 weeks when high values—2·82, 3·84, 4·95 mg./72 hr.—were obtained. There is unquestionably a very great increase in interstitial tissue in both rat and rabbit testis in response to iron-dextran loading. Unfortunately at the time it was not found possible to follow up this point.

Discussion (on testis)

The very low toxicity displayed by the iron-dextran complex made it possible for Nissim (1955) to administer doses to mice as high as 3 g. Fe/kg. He observed that in the course of about 10 weeks testicular atrophy resulted, with histological evidence of massive iron deposition in the proliferated interstitial tissue, severe degeneration of seminiferous tubules and absence of spermatozoa. In a later paper, Nissim (1956) attributes this result to the affinity of the interstitial cells for dextran without, however, demonstrating the presence of dextran in these cells following iron-dextran treatment. We have not been able to detect dextran histochemically in our material. There is reason to believe that testicular iron uptake will occur with any iron preparation which can maintain a stable high serum level over a sufficiently long period. It is likely also that it is the colloidal macromolecular form of the complex rather than its dextran component which predisposes to testicular deposition.

In our rats it was only at the highest level of dosage, 1650 mg. Fe/kg., and after a year that any abnormality of the testis was encountered. These degenerative changes are identical with the testicular effects observed in rats fed on diets deficient in vitamin E (Menschik, Munk, Rogalski, Rymaszewski and Szczesiak, 1949) and bear some resemblance to those reported in mice by Nissim (1955). Hodge, Maynard, Downs, Coye and Steadman (1956) encountered testicular atrophy in rats given ferric dimethyldithiocarbamate over 2 years. The same result was produced in guinea-pigs within a month by the daily intraperitoneal injection of ferrous gluconate in a dose of 20 mg. Fe/kg. (Telkkä, Kuusisto and Antila, 1956).

Observations on Other Organs

Two or more sections were taken from the muscles at the injection sites. The immediate reactions in the muscle following the injection of iron-dextran have been previously reported (Beresford *et al.* 1957). In the present work no gross lesions developed at the injection sites and, apart from heavy accumulation of siderophages, no histological abnormalities were seen in the muscle.

Histological sections were prepared from brain, pituitary, tongue, salivary glands, thyroid and parathyroid, trachea, oesophagus, lung, heart, aorta, stomach and intestines, pancreas, adrenal, spleen, ureters, bladder, prostate, uterus, ovary, thymus, lymph glands, bone marrow, rib, fat, nerve and skin. Much iron is present in the reticulo-endothelial system of the bone marrow, lymph glands and spleen and many of the siderophages contain a ceroid-like pigment similar to that in the liver. Small amounts of iron are consistently found in the acinar cells of the salivary glands and in the myocardial fibres of the rabbit; a few granules are seen in some pancreatic acinar cells of two of the rabbits. Parenchymal iron is not seen in those organs in the rat. In the brain, iron is seen only

in the choroid plexus. It is present in small and varying amounts in the stroma of all the organs but parenchymal abnormalities are not produced.

Tissue aerobic respiration studies were carried out in oxygen on slices of liver, kidney and heart in Ringer phosphate containing 0·2 per cent glucose. Slices of liver from iron-loaded rats and rabbits were too friable to withstand shaking in the Warburg apparatus. In rats of group D at 35 weeks there was no significant difference in Qo_2 from dextran controls. Similarly with kidney slices and rabbit heart slices—despite the presence of iron in the myocardial fibres—there was no change in the rate of respiration.

DISCUSSION

Effects of high doses of iron

These effects may be considered under two headings: the tissue response to the heavy doses of iron and the organ damage resulting from iron accumulation. The main features of the tissue response observed in these experiments have been the increased liver weight and the proliferation of the interstitial tissue of the testis. Gedigk and Strauss (1954) have shown that uptake of ferric hydroxide into the Kupffer cells evokes a response involving the synthesis of an organic matrix composed of mucopolysaccharide, lipid and protein, and possessing a specific binding capacity for colloidal iron. The amount of this material laid down in our animals is insufficient to account for the observed increase in liver weight; nor does it afford an explanation of the increased interstitial tissue in the testis. Both these changes are probably manifestations of a hyperplastic influence exercised by the massive iron deposits.

The organ damage is perhaps best regarded as the outcome of the sequence of changes depicted in Fig. 17. It will be seen that the species differences are attributed to the serum iron levels and that these are traced back to different activities of the reticulo-endothelial systems of the two species. A parallel can be drawn here between iron-dextran uptake from the circulation and physiological removal of chylomicra. Feigenbaum, Byers and Friedman (1954) have drawn attention to the important rôle played by the reticulo-endothelial system in the disposal of exogenous cholesterol. Here too there is remarkable difference between the powers of the rat and the rabbit to clear the plasma of the chylomicra. The fact that serum iron levels can attain such heights without some immediate reaction on the part of the animal is attributable to the stability of the iron-dextran complex. It is probable that the iron-binding capacity of the serum is not exceeded at any time.

Much emphasis has been placed in the past on the distinction between reticulo-endothelial and parenchymal deposition of iron. In fact, by a process of extraordinary extrapolation it has been assumed that the presence of parenchymal iron is the forerunner of haemochromatosis. This conclusion was drawn by Pinniger and Hutt (1956), who observed that in three rabbits treated with iron-dextran there was parenchymal iron deposition in the liver. In experimental iron loading there are very great species differences in the manner in which the same iron complex is handled by the body. Parenchymal iron in mice and guinea-pigs is distributed in a manner similar to that in the rat when treated with iron-dextran under the same conditions as our group A rats. The different manner in which the rabbit deals with iron-dextran may be traceable to a lower activity of its reticulo-endothelial system and perhaps to the different response of the

system to hormonal influences which to some extent govern the serum iron level (Cartwright, Hamilton, Gubler, Fellows, Ashenbrucker and Wintrobe, 1951). Species differences in organ reaction and vulnerability may also be partly a question of structure and function. Thus the rat kidney is known to be permeable

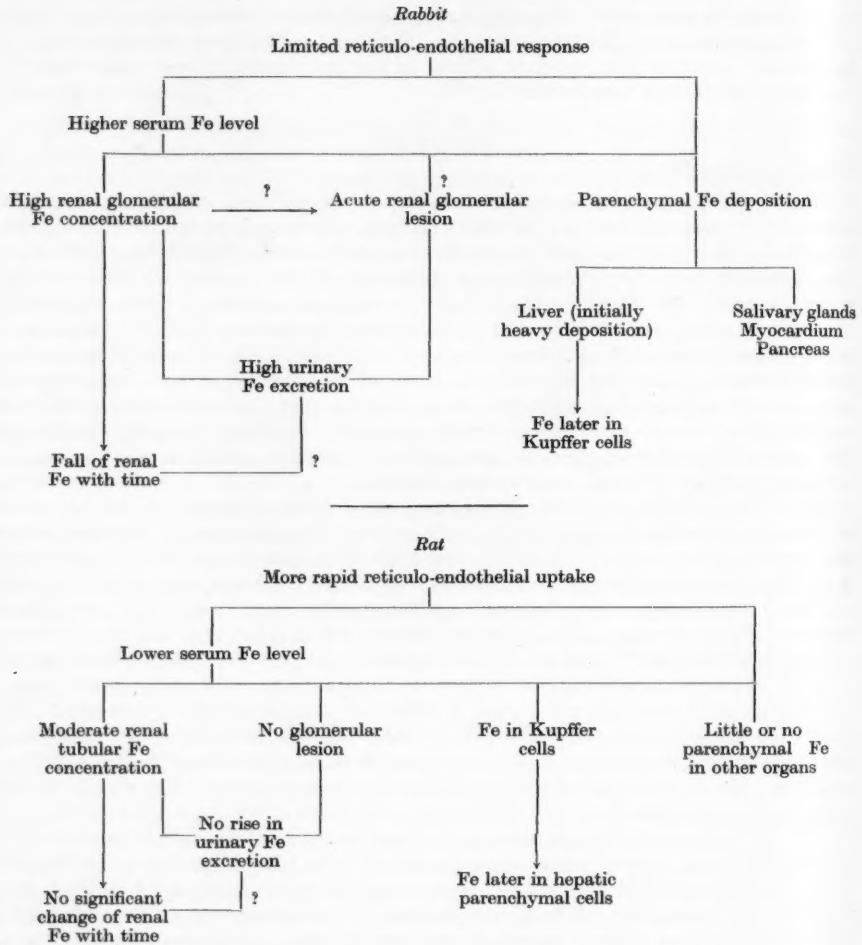
Rabbit
Limited reticulo-endothelial response
Higher serum Fe level
High renal glomerular Fe concentration
?
Acute renal glomerular lesion
Parenchymal Fe deposition
Liver (initially heavy deposition)
Salivary glands Myocardium Pancreas
High urinary Fe excretion
Fe later in Kupffer cells
Fall of renal Fe with time
?
Rat
More rapid reticulo-endothelial uptake
Lower serum Fe level
Moderate renal tubular Fe concentration
No glomerular lesion
Fe in Kupffer cells
Little or no parenchymal Fe in other organs
No rise in urinary Fe excretion
Fe later in hepatic parenchymal cells
No significant change of renal Fe with time
?
? Species effect
" Ceroid " pigment formation
Rat renal " lesion "

FIG. 17.—A schematic comparison of the manner in which the rat and the rabbit handle massive doses of iron-dextran complex (rat : 1650 mg. Fe per kg. ; rabbit : 825 mg. Fe per kg.).

to molecules such as the α- and β-globulins which appear in normal rat urine, whereas proteinuria is rare in the healthy rabbit. Hence the glomerular effects stemming from prolonged high serum iron level might well be less conspicuous in the rat kidney than they are in the rabbit kidney.

Influence of the dextran moiety of the complex

The stability of the iron-dextran complex in plasma and tissue fluid to a large extent determines its behaviour in the body. For example, the complex passes substantially unchanged through an ion exchange column, or the gastro-intestinal tract. Whereas large doses of saccharated oxide of iron cause precipitation in the lung capillaries, this does not occur with iron-dextran. It is only by virtue of its lack of dissociation in the plasma that iron-dextran can reach astronomical levels without causing the animal, or man, any discomfort. The dextran moiety must take credit for the stability of the complex but it is difficult to decide the exact point in the body at which iron and dextran part company. It is probable that this occurs at the primary sites of deposition in the reticulo-endothelial system, whence the iron is mobilized in a protein-bound form.

Dextran in tissue is readily stained by the alcoholic P.A.S. technique. Sections stained in this way were used with the intention of following the fate of the dextran moiety of the iron-dextran complex and the presumed conversion of the iron into haemosiderin. Gedigk and Strauss (1954) have however shown that haemosiderin in tissue sections is also stained by this technique due to the polysaccharide nature of the organic base of haemosiderin. They demonstrated that the iron is dissolved out of haemosiderin by treatment with dilute hydrochloric acid but can be returned again by treatment with colloidal iron as in Hale's technique. Iron-dextran complex is also dissociated by acid but recombination does not occur under the circumstances in which haemosiderin is reconstituted. By this means it is possible to distinguish between iron-dextran and haemosiderin, and we have shown that complete dissociation of iron-dextran with formation of haemosiderin occurs within a few days of injection.

Nissim (1956) holds the dextran moiety responsible for the accumulation of iron in the interstitial cells of the testis. In his classical studies on *intra vitam* staining, Cappell (1929) noted that the interstitial cells of the testis take up vital dyes to a considerable degree. We have observed the same effect with iron preparations not containing dextran in any form. It seems much more likely that it is the presence of iron circulating in high concentration as a stable complex which causes it to be accumulated by the testicular interstitial tissue.

Function of the organs affected by siderosis

Muir, Cappell and others have stressed that organ siderosis is not in itself a pathological state. This applies particularly to siderosis induced by exogenous parenteral iron, where no predisposing pathological cause for the siderosis is present. Nevertheless there exists a widespread reluctance to accept a clear-cut line of demarcation between haemosiderosis and haemochromatosis.

Polson (1933) found that six rabbits which had received dialysed iron intravenously over a period of 35–38 months all had negative galactose tolerance tests. At 44–46 months only one was unequivocally positive. In dogs given up to 1·0 g. Fe/kg. intravenously as the saccharated oxide and observed over a

period of 7 years, Brown *et al.* (1956) failed to find evidence suggestive of haemochromatosis and the following tests carried out at intervals were normal: fasting blood sugar, serum bilirubin, bromsulphthalein retention and serum protein.

The present experiments were accompanied by fairly extensive checks of function, particularly of the liver. It may be stated with confidence that no adverse effect on function was exercised by the massive load of iron permeating the livers of both rats and rabbits. The total quantity of body iron in these animals corresponds to an accumulation of 30–60 g. Fe in man, which is of the magnitude found in haemochromatosis. The period of study has, of course, been limited; but it would be anticipated that after well over a year any deleterious influence of the iron would have revealed itself.

SUMMARY

The consequences stemming from greater accumulations of iron than had hitherto been administered have been studied by means of intramuscular injections of iron-dextran complex in rats and rabbits. The total quantity of iron given corresponds to the content of body iron reported in most cases of human haemochromatosis.

Iron-dextran is absorbed rapidly into the circulation from the injection sites and is taken up chiefly by the reticulo-endothelial system. Small amounts of iron pass into parenchymal cells of the liver and kidney and into the interstitial cells of the testis. The tissue response was the same in rats and rabbits, consisting of a rapid increase in liver weight and a hyperplasia of interstitial cells of the testis.

Certain species differences in the sites and effects of iron deposition and accumulation are interpreted as consequences of the higher levels of serum iron which are attained in the rabbit.

Tests of hepatic, renal and testicular function, carried out over 60 weeks, failed to detect any constant departure from normality. It is concluded that in these animals no trend towards haemochromatosis has been detected.

Our thanks are due to the staffs of our respective departments for technical assistance, to Professor A. C. P. Campbell for his interest and advice, and to the Directors of Benger Laboratories, Ltd. for permission to publish the results of our investigations.

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THE IMMUNITY RESPONSES OF MICE TO INJECTIONS OF DIPHTHERIA AND TETANUS PROPHYLACTICS

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VERY little appears to be known about the immunity responses of mice to injections of antigens, though several reports on anaphylaxis in mice suggest that they are somewhat resistant to shock. Weiser, Golub and Hamre (1941), using egg-white as antigen, found that different strains and individuals of the same strain varied in their responses to active anaphylactic sensitization: the response was correlated with their precipitin-forming capacity. Solotorovsky and Winsten (1953) found that bovine albumen was a more efficient sensitizing agent after precipitation with alum, and Wheeler, Brandon and Petrenco (1950) showed that Freund's adjuvant greatly increased the sensitizing power of egg albumen in mice. These results suggest that precipitins may not be readily produced in this species in response to injections of fluid antigens.

Working with tetanus toxoids, Greenberg (1953) concluded that mice were unsuitable as test animals for the comparative assay of alum-precipitated and fluid toxoids. A single dose method was used and a large proportion (54 per cent) of assays on alum-precipitated toxoids were non-valid on account of non-parallelism of the dose-response curves with that of the standard fluid preparation. Fluid preparations appeared to give fair agreement, in relation to a fluid standard, when tested in mice or guinea-pigs but, even in some " satisfactory " assays giving parallel dose-response curves, alum-precipitated tetanus toxoids appeared far more potent in relation to the fluid standard, when tested in mice than in guinea-pigs. This suggests that the enhancement of antigenic efficiency brought about by alum-precipitation is more effective in mice than in guinea-pigs. The widely divergent results obtained in two separate tests on one preparation (13 and 99 times the potency of the standard) suggest also that great variability may occur in response not only of individuals but of different groups of mice.

We have carried out a detailed comparison of the responses of guinea-pigs and mice to a small number of prophylactics, the more important being purified diphtheria toxoid alone and adsorbed on aluminium phosphate, purified tetanus toxoid and alum-precipitated crude tetanus toxoid. The responses of mice to injections of diphtheria prophylactics is clearly of academic interest only, because the extreme resistance of this species to diphtheria toxin would render impracticable a test involving a challenge dose after immunization.

EXPERIMENTAL

Guinea-pigs of the same stock and weighing 350–450 g., were injected subcutaneously and were bled by cardiac puncture. Dealers' white mice weighing 18–20 g. were also injected subcutaneously and were bled out from the axillary vessels under anaesthesia at the end of the experiments. All animals received two injections separated by an interval of 28 days

and were bled 10 days after the second injection. Doses were usually administered to guinea-pigs in a volume of 1 ml. and to mice in 0·5 ml., due care being taken to avoid leakage.

Individual sera were titrated for diphtheria antitoxin by the guinea-pig intracutaneous method and for tetanus antitoxin by the mouse method. In order to obtain enough material for these individual titrations, the mouse sera were diluted with 4 volumes of saline before test. Since this procedure involved testing the lower value mouse samples at a higher dilution, it might be expected, from considerations of avidity, to yield apparently lower titres than the titration of undiluted sera ; a few pools were therefore tested undiluted and after diluting 1/5. After adjusting for dilution, the 1/5 samples never gave an apparent antitoxin titre less than half that of the undiluted serum, and were at times the same within the limits of error of the tests, which were performed at approximately 2-fold differences. The fact that titres of undiluted guinea-pig sera were compared with diluted mouse sera can therefore contribute very little to the enormous differences in the responses of the two species found in these experiments.

RESULTS

Diphtheria Prophylactics

Table I shows the antitoxin titres of animals after immunization with fluid diphtheria toxoid at various levels of dosage. When two doses, each 1 Lf or

TABLE I.—*Antitoxin Titres of Guinea-pigs and Mice 10 Days after the Second of Two Injections of Purified Diphtheria Toxoid, Separated by an Interval of 28 Days*

		Number of animals with antitoxin titres (units/ml.) :												
Animals	Dose (Lf)	> — < 0·005	0·005 0·01	0·01 0·02	0·02 0·05	0·05 0·1	0·1 0·2	0·2 0·5	0·5 1·0	1·0 2	2 5	5 10	10 20	Total
Guinea-pigs	0·2	6	0	0	2	1	1	0	1	3	0	1	—	15
	0·5	1	0	0	0	0	2	0	5	1	4	1	—	14
	1	—	—	—	—	—	—	—	1	7	7	—	—	15
	2	—	—	—	—	—	—	1	0	2	5	6	1	15
	5	—	—	—	—	—	—	—	1	0	7	5	2	15
Mice	2	9	1	0	0	1	0	1	—	—	—	—	—	12
	5	11	0	2	0	1	0	3	5	3	—	—	—	25
	20	18	0	1	1	1	2	2	5	1	—	—	—	31

more, were injected into guinea-pigs, all animals produced more than 0·2 unit/ml. and there was little individual variation within groups. There were however considerable numbers of non-responders (<0·005 unit/ml.) in all groups of mice, whatever the dosage. It is interesting to note the existence of small populations of good responders among the mice receiving 5 or 20 Lf doses, showing that it is possible for some mice to reach high titres. Nevertheless the Table shows that two doses of 0·5 Lf were more effective in guinea-pigs than two doses of 20 Lf in mice. Taking body weight into account, this dosage ratio becomes 1 : >800.

Table II shows the responses of animals to diphtheria prophylactic P.T.A.P. : this preparation contained a different batch of toxoid from that used in the preceding experiment. It is evident that the response of mice to adsorbed toxoid was far greater than that to the fluid material. The distribution and geometric mean antitoxic titres of the mice immunized with 0·8 Lf were comparable with those of guinea-pigs receiving 0·5 Lf. An additional group of mice, given two doses of 5 Lf, showed no improvement in response. It appears from Table II that about twice as much P.T.A.P. is necessary to overcome poor response in individuals of a group of mice than in a group of guinea-pigs. When body weight is taken into account, the factor becomes just over 40.

TABLE II.—*Antitoxin Titres of Guinea-pigs and Mice* 10 *Days after the Second of Two Injections of Aluminium-phosphate Adsorbed Diphtheria Toxoid (P.T.A.P) Separated by an Interval of* 28 *Days*

Animals	Dose (Lf)	Number of animals with antitoxin titres (units/ml.): > —	< 0·005	0·005–0·01	0·01–0·02	0·02–0·05	0·05–0·1	0·1–0·2	0·2–0·5	0·5–1·0	1–2	2–5	5–10	10–20	20–50	Total
Guinea-pigs	0·05	9		1	1	0	0	3	0	1	—	—	—	—	—	15
	0·1	5		0	1	1	0	0	1	3	2	1	1	—	—	15
	0·2	—		—	—	1	0	0	0	1	2	7	3	1	—	15
	0·5	—		—	—	—	—	—	—	—	—	4	7	2	—	13
	1·0	—		—	—	—	—	—	—	—	—	2	9	3	1	15
Mice	0·1	11		1	3	0	1	0	0	2	1	—	—	—	—	19
	0·2	10		0	1	1	0	0	0	6	2	5	—	—	—	25
	0·4	1		0	1	0	1	0	1	5	1	13	—	—	—	23
	0·8	—		—	—	—	—	—	—	1	3	8	6	4	—	22
	5	—		—	—	—	—	—	—	3	6	8	6	—	—	23

Comparison of Tables I and II shows that guinea-pigs required approximately twice as much fluid as adsorbed toxoid for the production of titres showing a small scatter, but mice would require more than 25 times as much. Higher doses than 20 Lf of fluid toxoid were not tried in mice: the large number of very poor responders and the considerable variation within groups suggested that further experiments would be unproductive. It is clear that mice are extremely refractory to immunization with fluid diphtheria toxoid.

A small experiment was done to determine whether the addition of *Haemophilus pertussis* vaccine would improve the response of mice to fluid toxoid. The toxoid was that used in the first experiment and vaccine was added to make a preparation containing 8×10^{10} organisms and 100 Lf/ml. diphtheria toxoid. Two levels of dosage differing 10-fold were used for each species. Table III shows

TABLE III.—*Antitoxin Titres of Guinea-pigs and Mice* 10 *Days after the Second of Two Injections of Diphtheria-pertussis Prophylactic, Separated by an Interval of* 28 *Days*

Animals	Dose (Lf)	Number of animals with antitoxin titres (units/ml.): > —	< 0·005	0·005–0·01	0·01–0·02	0·02–0·05	0·05–0·1	0·1–0·2	0·2–0·5	0·5–1	1–2	2–5	5–10	10–20	Total
Guinea-pigs	0·1	9		1	0	0	2	1	1	1	—	—	—	—	15
	1	—		—	—	—	—	—	—	—	—	7	7	1	15
Mice	0·5	3		2	0	1	2	2	1	4	3	4	1	1	24
	5	—		—	1	1	1	0	0	1	3	1	6	3	17

that inadequate responses resulted from doses of 0·1 Lf in guinea-pigs and 0·5 Lf in mice. When the dosage was increased 10-fold, variation among the guinea-pigs was extremely small. While the majority of mice receiving two doses of 5 Lf produced titres of more than 0·5 unit/ml., there was a downward tail to the distribution. These results, taken in conjunction with those in Tables I and II, support the general experience that *H. pertussis* vaccine increases the response of animals to diphtheria toxoid, but that it is not so effective an adjuvant as mineral carriers. This conclusion applies to both guinea-pigs and mice.

TABLE IV.—*Antitoxin Titres of Guinea-pigs and Mice* 10 *Days after the Second of Two Injections of Purified Tetanus Toxoid, Separated by an Interval of* 28 *Days*

Animals	Dose (ml.)	Number of animals with antitoxin titres (units/ml.): > —	< 0·05	0·05 0·1	0·1 0·2	0·2 0·5	0·5 1	1 2	2 5	5 10	10 20	20 50	50 100	100 200	Total
Guinea-pigs	0·0025		1	0	0	1	1	7	2	3	—	—	—	—	15
	0·005		—	—	—	2	1	2	6	2	1	1	—	—	15
	0·01		—	—	—	—	1	2	3	5	4	—	—	—	15
	0·02		—	—	—	—	—	1	6	8	11	4	—	—	30
	0·04		—	—	—	—	—	—	2	13	8	5	—	—	28
Mice	0·05		3	2	1	2	0	1	0	1	—	—	—	—	10
	0·1		5	0	3	1	2	1	2	2	2	4	1	—	23
	0·2		1	0	1	0	0	1	0	3	8	9	1	—	24
	0·5		2	0	0	0	1	3	2	3	14	14	6	1	46
	1·0		2	0	1	1	2	3	2	6	15	5	9	2	48

Tetanus Prophylactics

Table IV gives the antitoxin titres of guinea-pigs and mice after immunization with various doses of purified tetanus toxoid. The guinea-pig titres show the usual reduction in scatter on increasing the dose injected and there is no significant difference between the mean log responses of the groups receiving 0·02 or 0·04 ml. The mouse results are however interesting. The considerable number of good responders injected with 0·2 ml. had titres as high as or higher than the guinea-pigs injected with 0·02 ml. but the scatter was considerable. Increasing the dosage 2·5- or 5-fold did not reduce this scatter or alter the position of the peak of the main distribution: immunization with the two highest doses was carried out on two occasions, using groups of 25 mice each time. Larger doses than 1 ml. (which was actually administered as 0·5 ml. of a double strength dilution) were not given. The results in Table IV strongly suggest that little or no improvement in response would be likely to occur.

In comparing the effective dose for mice and guinea-pigs it can only be concluded that about 10–20 times as much was required to produce a large high-titre population in mice as to reach the flattening-out point of the dose-response

TABLE V.—*Antitoxin Titres of Guinea-pigs and Mice* 10 *Days after the Second of Two Injections of Alum-precipitated Tetanus Toxoid, Separated by an Interval of* 28 *Days*

Animals	Dose (ml.)	Number of animals with antitoxin titres (units/ml.): > —	< 0·05	0·05 0·1	0·1 0·2	0·2 0·5	0·5 1	1 2	2 5	5 10	10 20	Total
Guinea-pigs	0·001		13	—	—	—	—	1	—	—	—	14
	0·002		5	0	0	1	2	1	4	—	—	13
	0·005		—	—	—	1	0	6	6	2	—	15
	0·01		—	—	—	—	—	—	7	6	2	15
Mice	0·005		2	0	1	1	2	6	5	2	1	20
	0·01		3	2	0	0	1	1	4	4	3	18
	0·02		—	—	—	—	—	7	6	6	5	24
	0·1		—	—	—	—	—	1	3	16	4	24

The potency (combining power) of this preparation was about $\frac{2}{3}$ of that of the toxoid referred to in Table IV.

curve in guinea-pigs. When body weight is taken into account, the dose ratio becomes 200–400.

The results obtained with alum-precipitated tetanus toxoid are presented in Table V. They are similar to those for diphtheria prophylactic P.T.A.P. About twice as much alum-precipitated toxoid was necessary to produce a degree of immunity in mice comparable to that in guinea-pigs. A small scatter of titres resulted in both species when adequate dosage was given.

Diphtheria-tetanus Prophylactic

A mixture of purified diphtheria and tetanus toxoids was injected into guinea-pigs at two levels and into mice at one level of dosage. The results in guinea-pigs (Table VI) suggest that this was antigenically a reasonably well-balanced mixture:

TABLE VI.—*Antitoxin Titres of Guinea-pigs and Mice* 10 *Days after the Second of Two Injections of a Mixture of Purified Diphtheria and Tetanus Toxoids Separated by an Interval of* 28 *Days*

Number of animals with diphtheria antitoxin titres (units/ml.):

Animals	Dose (ml.)	< 0·001	0·001 – 0·002	0·002 – 0·005	0·005 – 0·01	0·01 – 0·02	0·02 – 0·05	0·05 – 0·1	0·1 – 0·2	0·2 – 0·5	0·5 – 1	1 – 2	2 – 5	5 – 10	> 10	Total
Guinea-pigs	0·005	1	3	5	1	3	0	0	2	—	—	—	—	—	—	15
	0·04	—	—	—	—	—	—	—	—	—	3	3	4	3	1	14
Mice	0·5	—	—	< 0·005 2	1	4	1	1	1	4	3	1	1	—	—	19

Number of animals with tetanus antitoxin titres (units/ml.):

Animals	Dose (ml.)	< 0·01	0·01 – 0·02	0·02 – 0·05	0·05 – 0·1	0·1 – 0·2	0·2 – 0·5	0·5 – 1	1 – 2	2 – 5	5 – 10	10 – 20	20 – 50	50 – 100	100 – 200	> 200	Total
Guinea-pigs	0·005	4	0	2	0	2	0	1	2	2	1	—	—	—	—	—	14
	0·04	—	—	—	—	—	—	—	—	—	1	5	8	—	—	—	14
Mice	0·5	—	—	—	—	—	—	2	0	0	2	8	2	4	1	—	19

doses of 0·005 ml. were insufficient for both components but good responses to both resulted from doses of 0·04 ml. When two doses, each 0·5 ml., were injected into a group of mice however the responses to the tetanus toxoid were fairly satisfactory but there was enormous variation between the diphtheria responses of individuals, with a suggestion of two populations. This result is hardly surprising since, in relation to guinea-pigs, mice are even more refractory to immunization with diphtheria toxoid than with tetanus toxoid. It does however emphasize the need for clinical trials of combined prophylactics. A preparation which produces good immunity to all components in one species may be unbalanced when used in a different species, including man.

DISCUSSION

It is clear that, compared with guinea-pigs, mice are extremely refractory to immunization with diphtheria and tetanus toxoids. It is possible that different strains vary in capacity to respond. Mice are comparatively much less refractory when these toxoids are precipitated by alum or adsorbed on aluminium phosphate.

The enhancement in antigenic efficiency resulting from precipitation and adsorption is much more marked in the immunization of mice than of guinea-pigs. This may account for the high proportion of non-valid assays of adsorbed tetanus toxoids when compared in mice with a fluid standard (Greenberg, 1953) and the finding that these adsorbed toxoids appeared to be unduly potent in relation to results in guinea-pigs: discrepancies might well be greater when only one dose is given.

In the early stages of this work we titrated sera from a limited number of mice 28 days after the first injection. Detectable antitoxin was present in the sera of only 3 of 24 mice given a dose of 0·5 ml. of fluid tetanus toxoid (see Table IV), in 10 of 24 mice receiving 5 Lf of diphtheria toxoid + *H. pertussis* vaccine (see Table III) and in 21 of 22 mice injected with 0·8 Lf of P.T.A.P. (see Table II). Groups injected with alum-precipitated tetanus toxoid were not tested after a single dose.

Barr, Glenny, Hignett, Randall and Thomson (1952) found no difference in the responses of babies to immunization with two doses, each 25 Lf of A.P.T., whether the first dose was given at the age of 6–10 days or at 6 weeks or more: babies with low maternally-conferred antitoxin were selected in order to avoid interference from passive antitoxin. Similar babies starting immunization with three doses, each 25 Lf of fluid purified toxoid, at the age of 6–10 days gave very poor responses. Results on a very small group given the same course but started at 7 months appeared to be better. New-born lambs, immunized with two doses, each 250 Lf of A.P.T., produced far higher antitoxin titres than other new-born lambs given three doses, each 250 Lf of fluid purified toxoid. A similar but even more marked effect was obtained in adult sheep.

It therefore appears that most animals respond much better to adsorbed than to fluid toxoids and that while this applies particularly to mice, it may be less evident in guinea-pigs than in most other species including man. The poor response of very young babies and of new-born lambs to fluid toxoid suggests that an improperly developed immunity-producing mechanism may respond adequately only when an adjuvant is present. That this is not the only factor is shown by the poor response of adult sheep.

It is possible that young babies and certain species of animals eliminate fluid toxoids at a very rapid rate, giving insufficient time for adequate stimulation of the antitoxin-producing mechanism. Alternatively, certain foreign proteins may be only feebly antigenic in some species, and may be tolerated, except in large doses, without bringing the defensive mechanism into action. If this were so, it seems probable that mineral carriers may play some other rôle in addition to retaining and slowly and continuously releasing the stimulating antigen.

Our results emphasize the need for clinical trials of prophylactics intended for the immunization of man and animals. The adoption of one type of standard reference preparation will ensure the use of efficient prophylactics only if the relative antigenic efficiency of adsorbed and fluid preparations is of the same order in the test animal as in man. In addition, it appears more logical to us that preparations intended for the active immunization of man in two doses should be assayed by a two-dose method in test animals. Greenberg and Benoit (1956) claim that "antigenicity of diphtheria and tetanus toxoids as measured in the guinea-pig is a direct indication of their effectiveness in man". These authors immunized babies with varying numbers of "immunizing units" of different

batches as determined by a single-dose assay in guinea-pigs. The following figures are taken from Tables II and III of their paper :

Batch	Toxoid units per dose	Total	Number of children with diphtheria antitoxin (units/ml.) : 0·008	0·016	0·032	0·064	0·128	0·256	0·512	1·024	2·048	4·096	8·192	Total
11–1	20·7	62	1	1	0	5	3	2	3	4	1	—	—	20
11–1	41·4	124	—	—	—	2	2	8	1	5	2	3	—	23
5–1	11	33	—	—	1	2	1	6	8	9	4	2	1	34

It is difficult to see that these figures, relating to two batches of combined P.T.A.P., justify their conclusion, and to accept that 3 injections, each 20·7 immunizing units, or even 3 injections each *41·4* immunizing units of 11–1 gave better immunity in babies than 3 injections, each *11* immunizing units of 5–1. Whether this is due to the normal error of the assay test, the variability in response of babies or the fact that three doses were given to the latter and one to the test animals, is a matter for speculation. Greenberg and Benoit draw attention to the lack of relationship between the Lf and the immunizing unit. In the particular groups whose responses are reproduced above, we suggest that the Lf was the better measure of efficiency in children : 11 immunizing units of 5–1 and 41·4 immunizing units of 11–1 each contained 10 Lf doses.

SUMMARY

Mice are far more refractory than guinea-pigs to immunization with diphtheria and tetanus toxoids. The difference in response between these species is greatly reduced by injecting these toxoids after precipitation with alum or adsorption on aluminium phosphate.

The bearing of this result and of species differences on the comparative assay of prophylactics is discussed, and the need for clinical trials of single and combined preparations emphasized.

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TUBERCULIN SENSITIZATION IN THE MOUSE

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FOLLOWING the observations of Lange (1923) and Boquet, Negre and Valtis (1927), it was for a long time held that tuberculin hypersensitivity did not develop in the tuberculous mouse. Later workers returning at long intervals to this study, however, have been almost equally divided for and against the early authors' view. Gerstl and Thomas (1941) confirmed the failure of the tuberculous mouse to react to intradermal tuberculin, and recently Holden, Seegal and Adams (1953) have failed to confirm Favour's (1947) demonstration of tuberculin cytotoxicity for tissue explants from tuberculous mice.

In contrast, Hart, Long and Rees (1952) noted that delayed systemic shock may be produced in tuberculous mice by intravenous tuberculin and this finding was confirmed by Kirchheimer and Malkiel (1953). Even more recently, Favour (1954) has failed to elicit skin sensitivity to tuberculin in tuberculous mice, even after adrenalectomy, yet Gray and Jennings (1955) have shown that tuberculin injected into the foot pad of tuberculous mice gives rise to a swelling maximal at 24–48 hours while similar injections in normal mice are without effect.

The response to re-infection has been less in doubt. Boquet *et al.* (1927) observed that a second tuberculous infection in mice evolved more rapidly, and Pagel (1940) showed that the response to tubercle bacilli inoculated intradermally into mice previously infected with virulent tubercle bacilli was modified in a number consistent with the development of hypersensitivity.

In mice infected by the injection of tubercle bacilli into the thigh, Selbie and O'Grady (1954) showed that subsequent infection of the other thigh of these animals gives a response having the characteristics of tuberculin-type hypersensitivity. Systemic tuberculin shock can be produced in animals infected in this way, and the transient reduction in size of the first lesion which accompanies the response to re-infection can be reproduced by the administration of tuberculin. This raised the possibility that the size of the primary lesion might be permanently reduced by repeated tuberculin and experiments to test this were undertaken. During the course of this work, certain changes were observed in the response to tuberculin which throw some light on tuberculin anergy in the mouse and bear analogy to those resulting from repeated tuberculin testing in man. It is this series of changes in the response to tuberculin which is now described.

METHODS AND MATERIALS

Inoculum

The animals were infected with the H37Rv strain of *Mycobacterium tuberculosis* grown for 21 days on Youman's modification of Proskauer and Beck's medium. The surface growth was harvested and a stable suspension prepared in Dubos' medium without the

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addition of albumen, by a combination of Griffith's tube homogenization and mechanical shaking. This suspension was standardized by opacity to contain about 10^9 organisms/ml.

Infection

Using a 1 ml. syringe and intradermal (gauge 26) needle, groups of 10 Strong A albino mice were inoculated in the left thigh as previously described (Selbie and O'Grady, 1954) with 0·1 ml. of this suspension or of the suspension diluted 1/100 with Dubos' medium without albumen. The animals, distinguished by dye marks, were housed in groups of 10. They were fed once daily on mouse cubes, and water was supplied as required. The course of the lesions arising from this infection was followed, as before, by measuring the greatest medio-lateral width of the thigh with sliding calipers.

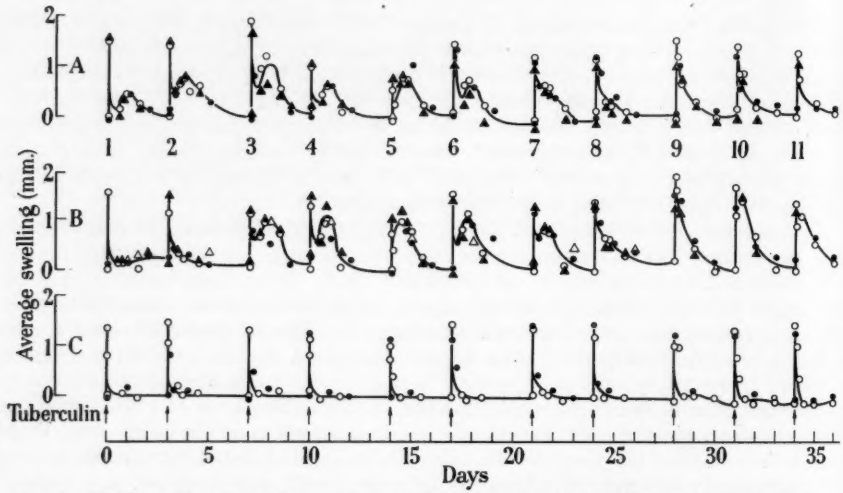

FIG. 1.—The average size of the swellings resulting from repeated tuberculin in (A) animals infected with inoculum diluted 1/100, (B) animals infected with undiluted inoculum, (C) normal animals. The different symbols represent different experiments.

Tuberculin treatment

Between the 27th and 35th days of this infection at which time the lesions were approaching their maximum size, half of the groups at each dose level began treatment with tuberculin, the remainder being kept as controls. At the same time, similar tuberculin treatment was instituted in a further group of 10 normal animals. An intramuscular injection of 0·1 ml. Old Tuberculin (at various times that supplied by four well-known manufacturers was used) diluted 1/5 with Sørensen's buffer pH 7·4, was made into the right thigh. The injections were repeated at alternating 3 and 4 day intervals (Mondays and Thursdays). In various experiments the thighs have been measured at the following time intervals: immediately after injection, 15, and 30 min., 1, 2, 5, 12, 20, 24, 36, 48, 60 and 72 hr. From these measurements the average difference in the size of the thighs in the treated and control groups has been calculated.

RESULTS

The response to serial injections of Old Tuberculin in normal mice and those infected with the undiluted and 1/100 inocula are summarized in Fig. 1. In normal animals (curve C, Fig. 1) Old Tuberculin gives rise to a swelling maximal at about 15 min. and subsequently declining logarithmically to zero. No measurable

swelling remains after 12 hr. However long the repeated injections continue, no significant change occurs either in the size or the form of this reaction.

The early responses in the two infected groups differ in an important respect. In the group receiving 1/100 dilution of the inoculum (curve A, Fig. 1), a bi-phasic response occurs, composed of a peak which is maximal at 15 min. and subsequently declines, followed by a second peak of much less rapid development and decline. The second peak is maximal between 20 and 24 hr. and the reaction following the first injection of tuberculin is regularly smaller than those to subsequent injections. In the groups receiving the larger infecting dose (curve B, Fig. 1), the reaction to the first two doses of tuberculin is apparently identical with that seen in the uninfected mice. The third injection, however, produces a bi-phasic response similar to that seen in the first infected group but differing in that the first response is of the same size as the subsequent reactions. Once this type of reaction with two components has appeared, the subsequent changes in the form of the response are identical in the two groups. These changes take place continuously but two stages in the process may be conveniently separated. In the first of these the 5, 12 and 20 hr. readings approximate closely to one another so that the overall response loses its bi-phasic appearance and comes to resemble a cubic curve (reaction 7, curves A and B, Fig. 1). In the second change, the size of the reaction at the 5, 12 and 20 hr. readings progressively declines instead of increasing as in the initial type of response.

In the animals receiving the smaller infecting inoculum, development and decay of the response to tuberculin soon occur so rapidly that the form of the reaction approaches that occurring in the non-infected group (reaction 11, curve A, Fig. 1) a state in which "desensitization" may be said to have occurred.

The reactions to intramuscular 1/5 tuberculin (dialysed and undialysed), saline, and 10 per cent glycerine (the concentration in 1/5 Old Tuberculin) are compared in Fig. 2.

DISCUSSION

In uninfected mice, the intramuscular injection of tuberculin gives rise to a definite reaction in that the swelling resulting from injection increases in size for about 15 minutes, while that resulting from saline progressively declines (Fig. 2). However long repeated injections into the thigh are continued, no significant change in the form of this reaction occurs. Comparison of this reaction with that produced by glycerine, and dialysed tuberculin (Fig. 2) suggests that the glycerine contained in Old Tuberculin makes a significant contribution to it. Raffel (1948) has shown that the very early reaction to Old Tuberculin in the skin of the infected guinea-pig can be greatly reduced by dialysis and has suggested that much of this response is due to glycerine. Boquet *et al.* (1927) have also drawn attention to the toxicity of glycerine for the mouse and discussed the possible rôle played by it in the systemic shock of this animal by Old Tuberculin.

The failure of this reaction to change in any way on repetition supports the finding that Old Tuberculin is non-antigenic (Smith, 1922). It is known, however, that protein sensitization of the mouse may require special conditions (reviewed by Weiser, Golub and Hamre, 1941) and the possibility that the development of Arthus-type hypersensitivity might not be recognized by the present method has not been excluded. The findings in the infected group, however, enable it to be said with certainty that tuberculin-type hypersensitivity does not develop in these circumstances.

In the infected animals, a response occurs which appears to consist of two separate components, the first of which develops rapidly, is maximal at about 15 minutes, and promptly declines. The question arises whether this rapid reaction represents the Arthus component of the tuberculin response. In magnitude, time relations, and form, it appears to be identical with the response obtained in uninfected mice. It seems unlikely, therefore, that it either is (or

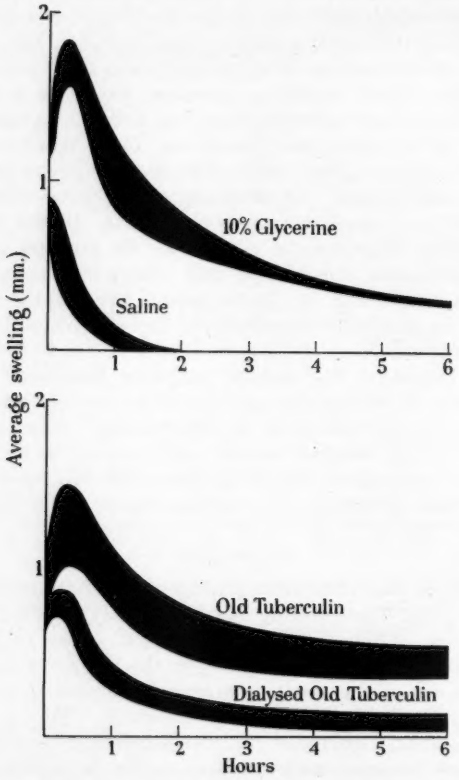

FIG. 2.—The limits of the size of the swellings produced by the injection of 10 per cent glycerine, saline, 1/5 Old Tuberculin, and dialysed 1/5 Old Tuberculin in numerous groups of normal animals.

conceals) an Arthus component. This is in keeping with the finding that the rapid component of the response to unheated tuberculo-protein cannot be elicited by Old Tuberculin (Raffel, 1948).

In the animals infected with the smaller inoculum, the first inoculation of tuberculin gives rise to a bi-phasic response in which the rapid component is followed by a reaction having the characteristics of tuberculin-type hypersensitivity. With repetition, the two components become increasingly difficult to separate until finally a reaction similar to the response of uninfected mice is

obtained. This may be explained as a progressive migration of the second reaction towards the first, the peak of the second reaction, while remaining the same size, occurring earlier with each reaction. The effect of this migration on the form of reactions resulting from such a combination of fast and slow components is illustrated in Fig. 3. The combined reaction, stage I of Fig. 3, should be compared with reaction 2, curve A and reaction 3, curve B, Fig. 1 ; stage II with reaction 7, curves A and B, Fig. 1 ; stage III with reaction 9, curves A and B, Fig. 1.

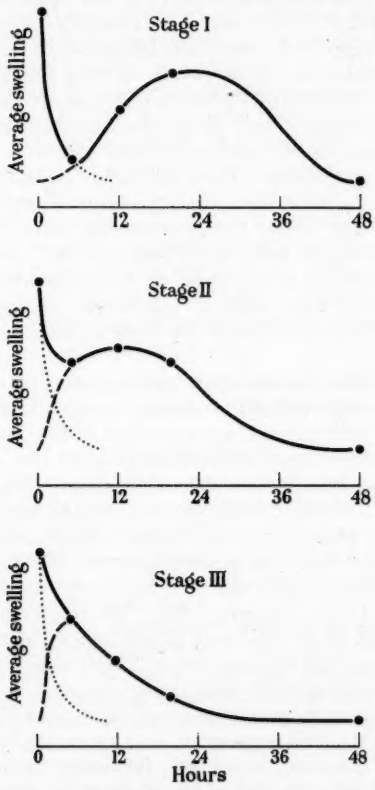

FIG. 3.—Combination of ..... fast component and – – – – – – slow component with maximum at Stage I, 24 hr. ; Stage II, 12 hr. ; Stage III, 6 hr.

In the animals receiving the larger inoculum the ultimate course of the reactions follows closely the pattern in the other infected group. There is a difference, however, in that the responses to the first two doses of tuberculin show no delayed component. At this time, when no delayed reaction is given to intramuscular tuberculin, animals similarly infected may be shown to be hypersensitive to systemic tuberculin and to tubercle bacilli injected into the thigh. The failure to respond to intramuscular tuberculin indicates, therefore, not absence of tuberculin hypersensitivity but the relative ineffectiveness of tuberculin administered in

this way. This emphasizes again that the readiness with which the manifestations of hypersensitivity may be elicited differs significantly both with the tissue tested and the method employed. The belief that the mouse does not develop tuberculin hypersensitivity has to a large extent arisen from preoccupation with the failure of this animal to respond to classical skin tests.

The third injection of tuberculin in the heavily infected animals gives rise to a response identical with that found in the group receiving the smaller inoculum. Once established, this reaction is followed by the same series of changes.

It appears that although tuberculin is incapable of sensitizing normal animals, once tuberculin sensitivity has been established by infection it can induce a further form of sensitization. As a result of this "tuberculin sensitization" a response is obtained to tuberculin where no response was obtained before, and the reactions to further tuberculin show progressive acceleration. There is an abrupt change from no response to the definitive response which remains at about the same *size* throughout the series. This supports the view first put forward by Fennyvessy and Freund (1914) that sensitization follows an all or none pattern. Walzer and Grove (1925), using pollen systems, have pointed out that intermediate stages of sensitization may occur but that they are unusual and unstable. In the present experiments a response of recognizably intermediate size was regularly obtained to the first dose of tuberculin in the animals receiving the smaller inoculum but this was always replaced on retesting by a response of the definitive size.

Mention must be made of the remarkable similarity between these reactions and those obtained on repeated skin testing in man. Lowenstein and Rappoport (1904) were the first to emphasize that repetition of the same skin dose (sometimes more than two doses were required) frequently gave rise to a reaction in cases in which no response was obtained to the first dose. This observation has been repeatedly confirmed for skin, conjunctival, and systemic tests (Cohn, 1907; Levy, 1908; Ellerman and Erlandsen, 1909; Bjornstad, 1941). Early workers also drew attention to the fact that in those cases in which a reaction was obtained, repetition of that dose often gave rise to a more rapidly developing and declining reaction (von Pirquet, 1907; Oppert, 1908; Mantoux, 1910). There is of course an important difference in the method of elicitation in the present experiments in that all doses of tuberculin were given into the same site. The opportunity for local antigen accumulation and sensitivity changes are greatly exaggerated compared with those occurring at separated skin sites in man, but the changes in the two groups nevertheless appear to bear close analogy.

Evidence has been discussed elsewhere (O'Grady, 1957) for the belief that in tuberculin-positive subjects short-lived responses to skin testing in man result from recent antigenic stimulation, either from an active tuberculous focus or from tuberculin administration. While this is supported in the mouse by the finding that increasingly rapid responses result from repeated tuberculin, this does not mean that the two stimuli are equivalent. In fact, although the power to manifest changes following repeated tuberculin depends on tuberculous infection, the two states of infection and tuberculin sensitization are to some extent antagonistic. Both in man and in the mouse it is in those minimally infected that the response to tuberculin and the changes to repeated testing are most readily elicited. It is known that with certain antigens, the inoculation of very large doses results in a state of "paralysis" in which further response to that antigen

cannot be obtained (Felton, 1949). It is possible that in heavily infected tuberculous animals, a state of "partial paralysis" is associated with the large antigenic mass and for this reason increased amounts of tuberculin are required to induce both the initial response and the cycle of changes which results from repeated testing.

SUMMARY

In normal mice, the injection of Old Tuberculin into the thigh produces a swelling maximal at about 15 minutes which subsides within 6 hours. In mice infected in the other thigh with *Myco. tuberculosis*, a similar injection of Old Tuberculin gives rise to a reaction having two components. The first of these appears to be identical with that seen in normal mice and reasons are given for attributing it to the glycerine contained in Old Tuberculin. The second component appears in about 6 hours, has its maximum at about 24 hours, and resolves within 48 hours. This is believed to represent true tuberculin hypersensitivity. In mice infected with a very large inoculum, Old Tuberculin produces at first a reaction indistinguishable from that in normal animals. When the injection of tuberculin is repeated at intervals of three or four days, a delayed component appears in the response of these heavily infected mice on the third dose. With continued repetition of the injections the peak of the delayed component occurs increasingly early in all the infected animals. The similarities between these changes and those seen on repeated skin testing in man are discussed.

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THE NATURE OF THE SPREADING EFFECTS OCCASIONED BY HYALURONIDASE IN SUBCUTANEOUS CONNECTIVE TISSUE

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PREVIOUS work on connective tissue studied *in vitro* (Day, 1952) suggested that the increased permeability occasioned by hyaluronidase was not due to liquefaction of a generally pervading "ground substance", but to the removal of hyaluronic acid from the interstices of a predominantly protein fabric arranged in membranes. The experiments to be described, which involve injections into the intact animal, both alive and dead, will be shown to give support to this view.

METHODS

In order to exclude any effect due to acute inflammatory response to the injection, the experiments to be described were performed on freshly killed animals as well as on living anaesthetized ones: McClean (1931) has shown that testicular extract is effective as a spreading agent in both dead and living tissue.

Technique of injection.—Injections were made with a glass pipette made from tubing of 6 mm. external diameter and 0·8 mm. thickness drawn out at an even diameter of 1·5 mm. for a distance of 3 cm. and pointed to 0·6 mm. at the end. The body of the pipette, some 10 cm. long, was graduated in volumes of 200 µl. Injections were made under air pressure supplied by a syringe connected by rubber tubing to the pipette and also to a U-tube water manometer. An appropriate volume of fluid having been sucked into the pipette, the skin of a mouse was plucked free of hair above the groin and punctured with a steel needle. The end of the pipette was inserted into the puncture and pushed gently upwards into the loose subcutaneous tissues of the flank. The pipette was kept parallel with the skin and as near to it as possible. It was then retracted for about 2 mm. in order to allow of an initial free flow into the small space so made. Injection was begun by pressing the syringe until the manometer registered the appropriate pressure. The same pressure was maintained throughout. *During the injection the needle track was firmly grasped between thumb and forefinger as close to the end as possible without occluding it.* This is a technical point of very great importance since it was found that at the low pressures used, the connective tissues resist penetration by water so that without this precaution there is progressive accumulation around the needle with final escape to the surface of the skin. In these circumstances the rate of inflow cannot of course provide any criterion of connective tissue permeability. All injections were made at a pressure of 15 cm. water. The standard observation was the time taken, measured with a stop-watch, for 400 µl. to flow into the tissues, but when flow was very slow 200 µl. was considered sufficient.

Anaesthesia and killing.—Ether was administered with an inhaler consisting of a test tube with ether-soaked cotton wool thrust into it for about 5 cm. Animals were killed mechanically. The back of the neck was pressed down firmly on the bench and the tail sharply pulled.

Dextran solutions.—The solution of smaller molecular weight (M.W.) was Benger's "Dextraven", a 6 per cent (w/v) solution in 0·9 per cent NaCl manufactured for intravenous therapy. The mean M.W. of such a solution is of the same order as that of the serum proteins. The pH of the solution used in these experiments was 6·0 measured colorimetrically. The solution of high M.W. was of purified native dextran provided by Benger Ltd. through

the kindness of their Medical Department. The mean M.W. was stated to be in the order of many millions. The solid dextran was dissolved by boiling to make a 1 per cent (w/v) solution in 0·9 per cent NaCl. The pH was 5·6.

Hyaluronidase.—Benger's " Hyalase " was used at concentrations stated in the text. This preparation is derived from bull testis.

Viscosity estimations were made with a U-tube viscometer in a water bath at 20° and at 30°.

RESULTS

Comparison of rate of inflow of native dextran with that of 6 per cent depolymerized " clinical " dextran

The figures in the following Table represent the mean of 10 observations in living and dead mice expressed in μl./min. followed, in brackets, by the standard deviation :

Native dextran		" Clinical " dextran	
Living	Dead	Living	Dead
90 (70)	50 (40)	100 (50)	80 (30)

There would appear to be no significant difference between the rates of inflow of these solutions.

Comparison of rate of inflow of the same solutions in the presence of hyaluronidase

Hyaluronidase was added to each solution at a concentration of 10 Benger units/ml. The mean rates of inflow were :

Native dextran		" Clinical " dextran	
Living	Dead	Living	Dead
240 (150)	250 (170)	1000 (370)	1000 (400)

It is clear that native dextran opposed the accelerating action of hyaluronidase to a far greater extent than did depolymerized " clinical " dextran.

Viscosity estimations of the two dextran solutions employed

The viscosities relative to distilled water at the same temperature were as follows :

	20°	30°
1 per cent (w/v) native dextran	4·73	4·60
6 per cent (w/v) " clinical " dextran	5·40	5·12

It is to be noted that at temperatures which are relevant to the conditions in which the injections were performed the solution of dextran of higher M.W. is distinctly less viscous than that of " clinical " dextran.

Hyaluronidase in concentrations of approximately 50 Benger units/ml. was added to the solutions in the viscometer tube. No change in the viscosity of either solution resulted.

Failure of the dextran solutions to neutralize the enzymic activity of hyaluronidase

It was thought possible that the observed counteraction of the accelerating effect of hyaluronidase upon inflow might be due to neutralization or inhibition of its enzymic activity. A mixture of hyaluronidase with each solution (130 Benger units/ml.) was therefore left overnight at room temperature. On the

following day the solutions were diluted tenfold with saline. Pieces of fresh mouse connective tissue were mounted as occluding membranes on the end of glass tubing in the manner described previously (Day, 1952). The capacity of a drop or two of each mixture to accelerate the flow of saline through such a membrane was tested. The tenfold or so acceleration characteristic of the action of hyaluronidase was observed in each case. Furthermore 400 μl. of the high polymer solution, diluted $\times$ 100 with saline was injected into living mice in the standardized conditions described above; the resulting flow rates 1200, 710, 920, 1000 and 3400 μl./min. were of the same order as those observed when hyaluronidase and saline were injected alone. It is to be concluded that neither dextran solution depresses the enzymic activity of hyaluronidase significantly.

DISCUSSION

It has been shown that a solution of very large macromolecules was far better able to slow down the increased rate of flow occasioned by hyaluronidase than was a solution of smaller macromolecules of the same chemical species. The w/v percentage concentration of the smaller molecules was six times that of the solution of the bigger and it was more viscous. It seems difficult to explain these facts except on the supposition that flow takes place through spaces of such a size that they can be blocked more readily by large macromolecules than by small ones.

This slowing effect did not depend on any reduction in the activity of the enzyme. The phenomenon accords, therefore, with the theory of the mode of action of hyaluronidase derived from experiments *in vitro* (Day, 1952), namely that the increased permeability brought about by this enzyme is due to removal of hyaluronic acid from the meshes of a protein fabric and that restoration of normal permeability by perfusion with large molecules is due to replacement of hyaluronic acid in the holes so created. The fact that molecular size rather than viscosity is the main factor in this restoration suggests that the spreading effects of the enzyme cannot be attributed solely to depolymerization of a generally pervading " ground substance ", but to the removal of its substrate from a specific situation in what has been shown to be a predominantly protein system (Day, 1949).

SUMMARY

When injected into subcutaneous connective tissue of mice, living or dead, a 1 per cent solution of purified native dextran of very great molecular weight counteracted the acceleration of flow occasioned by hyaluronidase to a greater extent than did a more viscous 6 per cent solution of partially depolymerized dextran.

This fact is in accordance with a conclusion previously reached that connective tissue is porous and that the pores are of macromolecular dimensions.

Any explanation of the spreading effect of hyaluronidase solely in terms of the reduction in viscosity of a generally pervading " ground substance " must be reconsidered in the light of this finding.

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THE INVASION OF THE BRAIN BY YELLOW FEVER VIRUS PRESENT IN THE BLOOD OF MICE

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ADULT mice are highly susceptible to intracerebral but relatively insusceptible to peripheral inoculation with most arthropod-borne viruses. Infant mice are highly susceptible to either type of inoculation, and the change-over to the adult response usually occurs during the second and third weeks of life (Bugher, 1941 ; Lennette and Koprowski, 1944). Attempts to define the "barrier" which develops in adult animals have not been very successful. During a study of the ability of peripherally inoculated yellow fever virus to invade the central nervous system of mice, results were obtained which help define this barrier, and in addition suggest the route by which the central nervous system is invaded.

MATERIALS AND METHODS

Standard diluent.—The standard diluent was 0·75 per cent bovine plasma albumin in saline buffered at pH 7·4.

Virus.—The Asibi strain of yellow fever virus was used in the form of serum from infected rhesus monkeys. Titres were found to be steady for at least a week when serum was stored in the frozen state at − 20°.

The French Neurotropic strain of yellow fever virus was in its approximately six-hundredth mouse passage, and virus was obtained as centrifuged mouse brain suspension. Suspensions were made in standard diluent and were used when fresh.

Mice.—The mice used were of Swiss stock, their ancestors originating from Carworth Farms, New York. Adults were inoculated when from 30–35 days old.

Infectivity titrations.—Serial tenfold dilutions of infective material were made in standard diluent, and each dilution inoculated (0·03 ml.) intracerebrally into 5 or 6 adult mice. Mice were observed for 12 days (French Neurotropic) or 16 days (Asibi) and end-points then calculated from deaths by the method of Reed and Muench (1938). Deaths which occurred on the first day (French Neurotropic) or during the first 4 days (Asibi) were regarded as non-specific and were not included in calculations. Death times were recorded from daily or twice daily observations.

In preliminary experiments, four different stock Asibi virus sera were titrated subcutaneously and intracerebrally in 3-day-old mice, and two of them intracerebrally in adult mice as well. Non-specific deaths were more common in infant mice, and two litters were therefore inoculated with each dilution. In Table I, where all titres are corrected to $\log_{10} LD_{50}$/0·03 ml., it can be seen that infant mice are rather more susceptible by the intracerebral than by the subcutaneous route, and even by the subcutaneous route they are about ten times as susceptible as are adult mice by the intracerebral route. Nevertheless, adult mice were used in titrations, because they were more readily obtainable and only half as many were required.

Organs were made up as 10 per cent suspensions in standard diluent, and the suspension considered as a 10^{-1} dilution in titrations, so that titres are expressed in LD_{50} per 0·03 ml. of solid tissue. Gall-bladders were discarded when adult mouse livers were removed, but this was not possible with infant mouse livers. Antibiotics, when used, were added so that each ml. of inoculum contained 1000 units of soluble penicillin and 1 mg. streptomycin.

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TABLE I.—*Titrations of Asibi Virus in Adult and Infant Mice*

	Titration method		
		3-day mouse	
	Adult, intracerebral	Subcutaneous	Intracerebral
Stock serum 1 . . .	5·0	5·7	6·3
„ „ 2 . . .	6·3	7·6	—
„ „ 3 . . .	—	2·3	3·2
„ „ 4 . . .	—	6·3	6·9

EXPERIMENTAL

The Growth of Yellow Fever Virus in Infant Mice

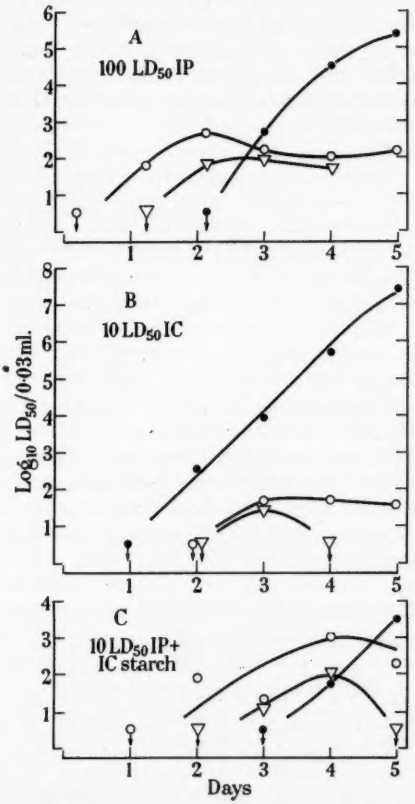
It was shown by Theiler (1930) that infant mice, unlike adults, are highly susceptible to peripherally injected yellow fever virus. When infants were inoculated intraperitoneally, virus first appeared in the brain by 48 hr. and pooled suspensions of liver, spleen and kidney were found to contain virus at 24 hr. but not later than this. Theiler thought that the virus present at 24 hr. might have been the residuum of the originally inoculated virus which was still present on the peritoneal surface of these organs. The experiments described below were done to see whether or not there was evidence for the visceral multiplication of virus in infant mice.

The effect of the route of inoculation.—Many litters of one-day-old mice were inoculated intraperitoneally with 100 LD_{50} of Asibi virus contained in 0·02 ml. of standard diluent. At intervals 5 infant mice, each from a different litter, were killed with ether and the bloods, livers and brains separately pooled and titrated. In the growth curves obtained (Figure A) it can be seen that the liver titre rose 1–2 days earlier than the brain titre, and then fell slightly while the brain titre continued to rise. Blood titres followed liver titres fairly closely.

In another experiment 10 LD_{50} of Asibi virus were injected intracerebrally (0·02 ml.) into one-day-old mice, and growth curves again obtained in the same way. Mice which were not sacrificed were observed, and the times of sickness and death recorded. It can be seen (Figure B) that blood and liver titres behaved much as in the previous experiment, although with the smaller dose inoculated a longer latent period preceded the rise in titres. Virus now appeared in the brain about a day earlier than in the liver. The median death time in 15 unsacrificed mice was 8·0 days.

Finally, about 10 LD_{50} of Asibi virus were injected (0·02 ml.) intraperitoneally into 14 litters of one-day-old mice. Immediately afterwards 0·02 ml. of 2 per cent starch solution was given intracerebrally to 11 of these litters, from which mice were taken at intervals and blood, liver and brain growth curves obtained in the usual way. The growth curves (Figure C) were similar to those obtained with intraperitoneal virus alone (Figure A), the longer latent period being attributable to the smaller dose inoculated. The intracerebral starch, therefore, had not affected the pattern of growth. Sickness and death was recorded in 21 unsacrificed mice as well as in the 3 litters which did not receive intracerebral starch. The median death time was 9 days in each group of mice, a day later than in the preceding experiment in which the same amount of virus was given intracerebrally. This difference in death time was confirmed when mice were inoculated either intraperitoneally or intracerebrally with an identical inoculum.

Four litters of one-day-old mice were given $10^{7\cdot0}$ LD_{50} intraperitoneally and their median death time (7 days) was a day later than for another 4 litters which had been given exactly the same dose intracerebrally.



FIGURE—Growth curves for Asibi virus in the liver (circles), blood (triangles) and brain (dots) of infant mice. Inocula and inoculation routes as shown. At each time interval five mice were sacrificed and titrations made on pooled material.

These experiments show that when Asibi virus is administered intraperitoneally to infant mice, 1–2 days' growth in the viscera precedes growth in the brain, even when the brain is traumatized by an injection of starch. When virus is deposited directly in the brain it begins to grow there before there has been visceral growth and death occurs about one day earlier. There is the same difference in death time even when large inocula are given. The greater part of an intracerebral inoculum spills over immediately into the blood stream (Cairns, 1950) and accordingly visceral growth occurred in the usual way after the intracerebral inoculation.

The route of invasion of the central nervous system.—An experiment was done in an attempt to find out which part of the central nervous system was first invaded by intraperitoneally injected virus. Many litters of 3-day-old mice were inoculated intraperitoneally with 100 LD_{50} of Asibi virus. At intervals 5 mice from different litters were killed with ether and different parts of their nervous systems removed, pooled and titrated in the usual way. The brains were divided into anterior and posterior parts so that the posterior part comprised the cerebellum, pons and medulla, and the spinal cords were taken from the thoracolumbar region. It was found (Table II) that although there was no detectable

TABLE II.—*The Growth of Asibi Virus in the Brains of Baby Mice after Intraperitoneal Inoculation*

Time (hr.)	Titres of the parts tested		
	Anterior brain	Posterior brain	Spinal cord
24	<1·0	<1·0	<1·0
53	<1·0	<1·0	<1·0
72	<1·0	<1·0	<1·0
78	1·6	1·0	1·5
96	3·0	1·6	1·0
120	4·0	3·5	3·8

virus in the 10 per cent suspensions at 72 hr., each of them contained small amounts of virus by 78 hr. Thenceforth the titres of the parts did not differ significantly, except at 4 days, and the importance of this particular difference is uncertain. It is concluded that Asibi virus injected intraperitoneally in infant mice either reaches each main part of the central nervous system at the same time, or alternatively spreads rather rapidly throughout the central nervous system from a single site of entry.

The Growth of Yellow Fever Virus in Adult Mice

Response to small inocula.—In the first experiment adult mice were inoculated subcutaneously (0·03 ml.) with 100 LD_{50} of Asibi virus. Five hours later, and then each day for 5 days, 5 or 6 mice were killed with ether and the pooled sera, livers, and brains each tested for virus by inoculating 3-day-old mice subcutaneously (0·03 ml.). Virus was at no time detected either in undiluted sera or in 10 per cent liver suspensions and brain suspensions.

In the next experiment, a similar dose was injected intracerebrally into adult mice, and at the same time intervals sera and livers were again tested for virus by inoculating infant mice ; on this occasion brains were titrated intracerebrally in adult mice. Detectable virus appeared in the brain by 24 hr. and the titre then gradually increased, reaching $10^{6·2}$ LD_{50}/0·03 ml. brain by the fifth day. No virus was detected in any of the 10 per cent liver suspensions but there was evidence that small amounts were present in serum on the second and fifth days. Some of the infant mice inoculated with these undiluted sera sickened or died after from 10 to 12 days and their pooled, seitz-filtered brain suspensions killed adult mice within 8–12 days on intracerebral inoculation.

In two more experiments where similar amounts of virus were injected intravenously and intraperitoneally into adult mice, and liver and serum pools tested once more in infant mice, virus was not isolated from liver pools and there was no evidence of viraemia.

These experiments show that when a small dose of Asibi virus is administered either intravenously, intraperitoneally, or subcutaneously into adult mice, virus is not at any time detectable in the liver or serum. There was probably a very low grade viraemia following intracerebral inoculation but this might have been due to a leakage of virus from the brain. Thus, while virus multiplies in the brain following intracerebral inoculation there is little or no visceral multiplication, whatever the route of inoculation.

Attempt to adapt virus to grow viscerally in adult mice.—Because Asibi virus grew viscerally in infant but not in adult mice it was thought possible that a series of passages in the viscera of infant mice might increase the ability of virus to grow viscerally in adult mice. About $10^{4\cdot0}$ LD_{50} of Asibi virus were injected intraperitoneally into 3 litters of one-day-old mice and 3 days later, when virus is known to be present in liver and blood (Figure A and C), 5 mice were sacrificed and the pooled livers suspended in standard diluent and centrifuged. After the addition of antibiotics the suspension was titrated intracerebrally in adult mice and injected intraperitoneally into another series of one-day-old mice. Livers from these mice in turn were dealt with in the same way so that a viscerotropic passage series was initiated. Later in the series mice were inoculated subcutaneously as well as intraperitoneally, and passages were made at 4-day intervals; on five occasions livers contained very little virus at the time of sacrifice and brains from mice sick at 9 days were then passaged. After 32 passages, the peak liver titres attained in infant mice had not altered, and remained less than $10^{3\cdot0}$ LD_{50}/0·03 ml. liver. Nor had there been any change in the response of adult mice to small intraperitoneal inoculations, for they were still insusceptible and still failed to develop viraemia on the third day. The tests for viraemia were made in two separate experiments by making a pool of serum from 3 mice and injecting it undiluted into adult mice by the intracerebral route.

There is therefore no evidence that the visceral passage of Asibi virus in infant mice increases its ability to grow viscerally in adult mice.

Response to large inocula.—It was found that adult mice were susceptible to the peripheral administration of Asibi virus if the dose was large enough. The results of many tests showed that a large proportion of adult mice died when inoculated intraperitoneally or intravenously with more than about $10^{6\cdot0}$ LD_{50} of Asibi virus. It was found that about $10^{5\cdot0}$ LD_{50} were lethal if the brain was pierced by a needle at the time of the intraperitoneal inoculation and there was a similar, although less well marked effect, when the spinal cord was pierced in the same way.

Adult mice are more susceptible to the intraperitoneal inoculation of the French Neurotropic strain of yellow fever virus if the brain is damaged at the same time (Sawyer and Lloyd, 1931). It was shown by Goodner and Smithburn (1951) that this occurred even when an intracerebral injection of 2 per cent starch solution was given 3 hr. before or ½ hr. after the virus inoculum. If brain trauma acts by directly exposing central nervous tissue to circulating virus it is not easy to understand why tissues remain exposed for at least 3 hr. It seemed possible, in the light of this finding, that the trauma acts indirectly by locally increasing the permeability of the blood-brain barrier. A series of experiments was therefore done to test this possibility. Randomized groups of 15–20 adult mice were inoculated intraperitoneally with large doses of Asibi virus and at the same time the brains of one group were pierced with a needle. A control group

of mice received nothing except the virus inoculum and other groups were given treatments in attempts to produce local dilatation of the blood vessels in the central nervous system (Trueta and Hodes, 1954). These treatments were: an intramuscular injection of a sterile emulsion of oil of turpentine, the production of a skin-muscle burn in the scalp or on the leg, and the production of fatigued limbs by inducing mice to swim for 40 min. by immersing them in lukewarm water. The results showed that although mice were more likely to die of yellow fever if the brain was pierced with a needle at the time of the virus injection, none of the other treatments had this effect. A similar experiment was done using the French Neurotropic strain, and the results were the same. This strain of virus, like the Asibi strain, was found to invade the undamaged brain when large enough doses were injected intraperitoneally.

Both these strains of virus, therefore, tend to invade the brain of adult mice when large intraperitoneal or intravenous doses are given. This tendency is increased when the brain or spinal cord is directly damaged, but other treatments which might indirectly alter the permeability of the blood-brain barrier are ineffective.

In investigating the ability of peripherally injected virus to invade the brain of adult mice it seemed important to know how long virus stays in the blood. An experiment was therefore done in which $10^{6 \cdot 7}$ LD_{50} of Asibi virus were injected intravenously into 6 adult mice, and at intervals these mice were bled from their tails and the pooled blood titrated. At 1 hr. and at 3 hr., mice were anaesthetized by the intraperitoneal injection of a barbitone-chloral hydrate mixture, then killed by exsanguination from the heart, and thorough nasopharyngeal washings obtained in standard diluent. These washings were centrifuged and after the addition of antibiotics injected intracerebrally into adult mice. The results (Table III) show that nearly all virus had disappeared from the blood within 10

TABLE III.—*The Disappearance of Intravenously Injected Asibi Virus from the Blood of Adult Mice*

Time after inoculation	Blood titre	Fate of mice inoculated with nasopharyngeal washings
0	4·7 (calculated)	—
10 min.	2·9	—
1 hr.	1·0	s s s s s s s s s s
3 „	—	s s s s s
8 „	<1·0	—

Each s indicates survival of an inoculated mouse.

min., and none was detectable in tenfold diluted blood of the 3 remaining mice at 8 hr. No mice inoculated with nasopharyngeal washings died, and it is concluded that little or no virus was present on the nasal mucosa at the times tested. Virus was injected intravenously in this experiment and disappeared from the blood so rapidly that there would probably be little chance of detectable viraemia being established after intraperitoneal inoculation where there was a gradual release of virus into the blood. Nevertheless, virus may enter the brain after large inocula, whether they are given intraperitoneally or intravenously and it may be that the extent and duration of viraemia is not of great importance in this connection.

In another experiment in which approximately $10^{7.0}$ LD_{50} of virus were injected intraperitoneally into 6 adult mice, and daily pooled tail blood samples diluted tenfold and tested in adult mice, viraemia was not detected. Two mice were sacrificed on the fourth day, and their livers were pooled and the titre found to be $10^{1.1}$. Two of the remaining 4 mice died, on days 10 and 11. On another occasion 2 mice were given $10^{5.0}$ LD_{50} intraperitoneally and their pooled liver titre on the fourth day was $10^{2.0}$. It seems possible, therefore, that the liver plays a part in removing virus from the blood.

Comparison of the ability of neurotropic and pantropic virus strains to infect adult mice by the nasal route.—Several viruses have been shown to invade the central nervous system from the nasal mucosa *viâ* the olfactory tracts. Sometimes these results have been difficult to interpret because neurotropically adapted strains of virus were used. The Asibi and French Neurotropic strains were titrated intracerebrally and intranasally in adult mice and the results (Table IV)

TABLE IV.—*Comparative Titrations of French Neurotropic and Asibi Strains in Adult Mice by Intracerebral and Intranasal Routes*

Virus	Titres	
	Intracerebral	Intranasal
Asibi	5·0	<0*
French Neurotropic	7·0	3·1

* No mice receiving undiluted virus died.

show that, whereas for French Neurotropic $10^{4.0}$ intracerebral LD_{50} are equivalent to an intranasal LD_{50}, more than $10^{5.0}$ intracerebral LD_{50} of Asibi are required. The neurotropically adapted strain therefore infects more readily than the pantropic strain when administered intranasally.

DISCUSSION

It has been shown that when the Asibi strain of yellow fever virus was injected intraperitoneally into infant mice there was a period of 1–2 days' visceral growth as shown by a rise in liver and blood titres, before virus was detectable in the brain. When virus was injected directly into the brain it multiplied there without this period of delay, and death occurred approximately one day earlier. It is suggested that the period of visceral growth is necessary before peripherally introduced virus can reach the central nervous system. Adult mice would then be insusceptible to peripherally introduced virus because, as has been shown, this visceral growth probably does not occur. The fact that they are susceptible to exceedingly large peripheral inoculations will be discussed later. Thus, the " barrier " which develops in adult mice would involve the loss of cells which support the visceral multiplication of virus. It is noteworthy that Semliki Forest virus, one of the few arthropod-borne viruses which readily kill adult mice on peripheral inoculation (Smithburn and Haddow, 1944), has been shown to undergo a period of visceral growth before it appears in the brain. When $10^{3.0}$ LD_{50} of this virus were injected intraperitoneally in adult mice a rise in blood titre occurred 1–2 days before a rise in brain titre (M. C. Williams, personal communication).

In the experiments where adult mice were given small extraneural inocula there was no evidence for the multiplication of virus, in spite of the fact that the tests were made particularly sensitive by the use of infant mice. Injected virus, however, was shown to disappear rapidly from the blood of adult mice, and thus the rate of entry of virus into the blood must also be rapid if viraemia is to be detected. It is therefore conceivable that virus was released into the blood in these experiments but was removed too rapidly for virus to be detected. If there had been visceral multiplication it is unlikely that it took place in the liver, for virus was never detected in this organ. Moreover, even if virus had been released into the blood from other organs or tissues, the liver might have been expected to contain virus which it had taken up from the blood ; it was shown in another experiment that virus was still present in liver suspensions 4 days after a large intraperitoneal inoculation. It is therefore thought improbable that visceral multiplication occurs in adult mice.

Why should 1–2 days' visceral multiplication be necessary before Asibi virus can reach the central nervous system of infant mice? Possibly in infants the viraemia lasts long enough for virus to be excreted on the nasal mucosa, and the brain is then invaded by way of the olfactory tracts but, for the reasons given below, it is thought unlikely that the nasal mucosa is important. If, however, virus grew in the endothelium of capillaries in the central nervous system and was then released both back into the blood and into the adjacent nervous tissue, it would have reached nervous tissue as a direct consequence of visceral growth. It may be that in infant, but not in adult mice, these capillary endothelial cells support virus multiplication. In the growth curve experiments with infant mice, liver titres were consistently slightly higher than blood titres, so that multiplication may therefore occur in the liver but it would not necessarily occur exclusively there.

Two of the routes by which viruses present in the blood might reach the central nervous system were clearly envisaged many years ago by Hurst (1936) and by King (1939). Either virus crosses the blood-brain barrier at one or more sites, or it reaches the brain by way of the olfactory tracts after being excreted on to the nasal mucosa. Clearly the question is of great importance in understanding the occurrence of encephalomyelitis in man following infection with arthropod-borne viruses or poliomyelitis virus, but experimental evidence has sometimes been difficult to interpret. For one thing, neurotropically adapted rather than recently isolated strains have often been used and, as has been shown above for the French Neurotropic strain of yellow fever, such strains may be unusual in their ability to infect by the nasal route. The fact that after the peripheral inoculation of infant mice, Asibi virus appeared in each main part of the central nervous system at about the same time, suggests that the nasal mucosa is not important in this instance. Adult mice are so insusceptible to intranasal inoculation that, even if large amounts of virus were excreted on to the nasal mucosa, it is unlikely that it would then reach the brain.

If, as is suggested, virus reaches the central nervous system of infant mice by " growing through " blood vessels (Hurst, 1936), two other experimental findings become easier to understand. First, a widespread release of virus from blood vessels into the substance of the central nervous system would initiate growth far more effectively than an intracerebral inoculation which merely flooded the needle track and the cerebrospinal fluid with virus (Mims, unpublished

observations). It might therefore be expected that virus would reach all available cells and kill the mouse more rapidly, when it entered central nervous tissue at many points from blood vessels. There is evidence that this is, in fact, so, because although after an intraperitoneal inoculum virus appeared in the brain 2–2½ days later than after an intracerebral inoculum of the same size (Figure B and C), the death time was delayed only by one day. Second, it was shown that intracerebral starch did not significantly alter the period of visceral growth which preceded the growth of Asibi virus in the brain of infant mice. This would be expected if, as a result of visceral multiplication there was a widespread release of virus into the brain which made the very small local entry of virus which might have taken place at the site of the intracerebral starch injection unimportant by comparison.

It is not easy to explain why adult mice are susceptible to large doses of Asibi virus by peripheral routes of inoculation. It was shown that even after intravenous inoculation virus disappears very rapidly from the blood, and there was no detectable virus on the nasal mucosa at the times tested. It is possible that when large doses are given there is enough virus present in the blood for a long enough period for it to enter the central nervous system directly, as a result of the changes in capillary permeability which may be assumed to occur in normal adult mice. In passing the blood-brain barrier in this way virus would " leak " through mechanically, in contrast to the " growth through " which, it is suggested, occurs in infant mice. Since it is possible that different viruses use different routes or a given virus more than one route, these experimental findings should, strictly speaking, be applied only to the virus-host system used in the experiments.

SUMMARY

It has been shown that when small doses of the Asibi strain of yellow fever virus are administered intraperitoneally to infant mice, a period of 1–2 days' visceral multiplication precedes the appearance of virus in the brain. When virus is given intracerebrally, it appears in the brain about a day earlier than in the viscera and death now occurs a day earlier. It is suggested that virus reaches the brain from the periphery as a result of the preceding visceral multiplication, possibly by multiplying in the endothelium of capillary blood vessels in the central nervous system. There is no visceral multiplication in adult mice and it is to this that their insusceptibility is attributed.

When virus is given intraperitoneally to infant mice, it appears in each main part of the central nervous system at about the same time. This is taken as evidence that virus reaches the central nervous system directly from blood vessels.

Adult mice are susceptible to the peripheral inoculation of very large doses of Asibi virus. Smaller doses are sufficient if the central nervous system is traumatized at the time of the inoculation, but treatments designed to alter capillary permeability in the central nervous system do not have this effect.

Intravenously injected virus is removed very rapidly from the blood of adult mice.

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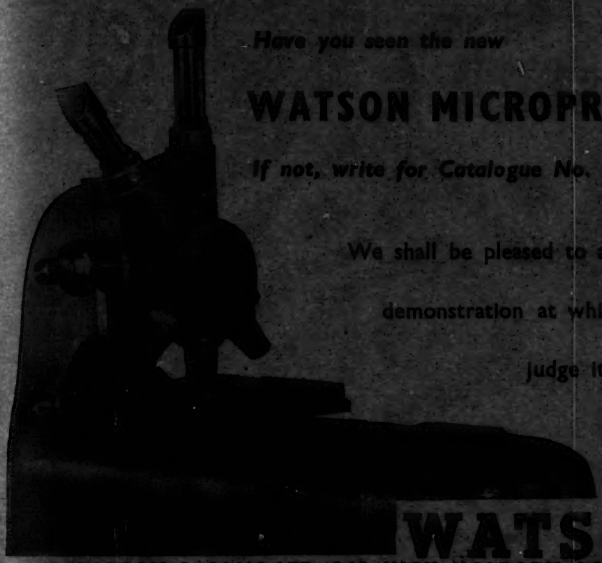

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